Vol. XIV. No. 2. JUNE, 1960

THE BRITISH JOURNAL OF CANCER

(The official journal of the British Empire Cancer Campaign)

EDITOR
R. W. Scarff

ASSISTANT EDITOR
A. C. Thackray

ADVISORY EDITORS

Sir Samuel P. Bedson
Sir Stanford Cade
Sir Charles Dodds
Malcolm Donaldson
C. E. Dukes
L. H. Gray
A. Haddow
W. D. Newcomb
Sir Heneage Ogilvie
Percy Stocks
B. W. Windeyer

Copyright © 1960

LONDON
H. K. LEWIS & CO. LTD.
136 GOWER STREET, W.C.1

Published Quarterly
Annual Subscription, post free, Four Guineas
Single copies, One Pound Five Shillings net

EDITORIAL.

Scope of the Journal.—For the publication of original communications concerning the causes, distribution, symptoms, pathology and treatment of malignant diseases and allied conditions. Papers upon clinical, bacteriological, biochemical, pharmacological, physiological, radiological, serological, and other subjects therefore would be acceptable when related to this subject.

Forwarding Papers and Correspondence.—Papers should be forwarded to Professor R. W. Scarff, The Middlesex Hospital, London, W.1.

Subscriptions and business correspondence are received by the publishers, Messrs. H. K. Lewis & Co. Ltd., 136, Gower Street, W.C.1.

Date of Publication.—The Journal is published quarterly in March, June, September and December.

Rapidity of Publication.—All papers accepted for publication will be printed, so far as is possible, in the next following issue of the Journal, if received before the first day of the month preceding publication. Long papers, or papers with illustrations, may, however be delayed if not received earlier.

Reprints.—25 reprints will be supplied free to authors who are registered as personal subscribers to the Journal. Additional copies must be ordered from the Publishers before the issue is printed. Prices on application.

BRITISH JOURNAL OF CANCER

VOL. XIV JUNE, 1960 NO. 2

DEVELOPMENT OF CARCINOMA OF THE CERVIX UTERI

Observations Resulting from Cytological Examination of 10,000 Cervical Smears

M. M. BODDINGTON, R. H. COWDELL and A. I. SPRIGGS

From the Department of Pathology, United Oxford Hospitals.

Received for publication March 28, 1960

In 1928 Papanicolaou in New York and Babès in Bucharest independently discovered that carcinoma of the uterus sheds identifiable cells into the vaginal fluid. This finding was later developed by Papanicolaou and others into an important diagnostic method which has now been widely adopted. The cytological diagnosis of carcinoma of the uterus has accumulated an enormous literature, quite out of proportion to the limited information which can be extracted from the examination of vaginal or cervical smears.

It soon became clear that the new method reveals certain abnormalities of the cervical epithelium other than invasive squamous carcinoma. There is, in fact, a whole range of cytological changes corresponding to the histological appearances labelled " atypical hyperplasia " and " carcinoma *in situ* ", and the interpretation of smears immediately became involved in the controversial problem of precancerous change (Ayre, 1952*a*). Since the histological diagnosis is the universally accepted yardstick by which the accuracy of cytological diagnosis is assessed, we have the situation of the blind leading the blind.

The results of experimental work on carcinogenesis show that invasive cancer develops in the course of a series of cellular transformations which are probably discontinuous. After invasion has begun, further transformations may continue to occur, producing increases in malignancy (Foulds, 1958). Among other sites, this discontinuous transformation has been demonstrated experimentally in the cervix uteri of the mouse (von Haam and Scarpelli, 1955; Reagan and Wentz, 1959). All the evidence from human material supports the same concept. In one cervix a series of lesions may be found from basal cell hyperplasia or squamous metaplasia up to invasive carcinoma (Howard, Erickson and Stoddard, 1951; Gusberg and Moore, 1953; Carson and Gall, 1954).

Direct evidence of the precancerous nature of " carcinoma *in situ* " in the human cervix is of two kinds. In the *retrospective* study of Galvin, Jones and Te Linde (1952), out of 13 patients with carcinoma of the cervix who had undergone biopsy one to seventeen years before, a review of the old material revealed " carcinoma *in situ* " in 11. Many *prospective* studies have shown progression of " carcinoma *in situ* " to invasive carcinoma, often after a lapse of many years (Younge, Hertig and Armstrong, 1949; Gusberg and Moore, 1953); but this progression

EDITORIAL.

Scope of the Journal.—For the publication of original communications concerning the causes, distribution, symptoms, pathology and treatment of malignant diseases and allied conditions. Papers upon clinical, bacteriological, biochemical, pharmacological, physiological, radiological, serological, and other subjects therefore would be acceptable when related to this subject.

Forwarding Papers and Correspondence.—Papers should be forwarded to Professor R. W. Scarff, The Middlesex Hospital, London, W.1.

Subscriptions and business correspondence are received by the publishers, Messrs. H. K. Lewis & Co. Ltd., 136, Gower Street, W.C.1.

Date of Publication.—The Journal is published quarterly in March, June, September and December.

Rapidity of Publication.—All papers accepted for publication will be printed, so far as is possible, in the next following issue of the Journal, if received before the first day of the month preceding publication. Long papers, or papers with illustrations, may, however be delayed if not received earlier.

Reprints.—25 reprints will be supplied free to authors who are registered as personal subscribers to the Journal. Additional copies must be ordered from the Publishers before the issue is printed. Prices on application.

BRITISH JOURNAL OF CANCER

VOL. XIV JUNE, 1960 NO. 2

DEVELOPMENT OF CARCINOMA OF THE CERVIX UTERI

Observations Resulting from Cytological Examination of 10,000 Cervical Smears

M. M. BODDINGTON, R. H. COWDELL and A. I. SPRIGGS

From the Department of Pathology, United Oxford Hospitals.

Received for publication March 28, 1960

In 1928 Papanicolaou in New York and Babès in Bucharest independently discovered that carcinoma of the uterus sheds identifiable cells into the vaginal fluid. This finding was later developed by Papanicolaou and others into an important diagnostic method which has now been widely adopted. The cytological diagnosis of carcinoma of the uterus has accumulated an enormous literature, quite out of proportion to the limited information which can be extracted from the examination of vaginal or cervical smears.

It soon became clear that the new method reveals certain abnormalities of the cervical epithelium other than invasive squamous carcinoma. There is, in fact, a whole range of cytological changes corresponding to the histological appearances labelled "atypical hyperplasia" and "carcinoma *in situ*", and the interpretation of smears immediately became involved in the controversial problem of precancerous change (Ayre, 1952*a*). Since the histological diagnosis is the universally accepted yardstick by which the accuracy of cytological diagnosis is assessed, we have the situation of the blind leading the blind.

The results of experimental work on carcinogenesis show that invasive cancer develops in the course of a series of cellular transformations which are probably discontinuous. After invasion has begun, further transformations may continue to occur, producing increases in malignancy (Foulds, 1958). Among other sites, this discontinuous transformation has been demonstrated experimentally in the cervix uteri of the mouse (von Haam and Scarpelli, 1955; Reagan and Wentz, 1959). All the evidence from human material supports the same concept. In one cervix a series of lesions may be found from basal cell hyperplasia or squamous metaplasia up to invasive carcinoma (Howard, Erickson and Stoddard, 1951; Gusberg and Moore, 1953; Carson and Gall, 1954).

Direct evidence of the precancerous nature of "carcinoma *in situ*" in the human cervix is of two kinds. In the *retrospective* study of Galvin, Jones and Te Linde (1952), out of 13 patients with carcinoma of the cervix who had undergone biopsy one to seventeen years before, a review of the old material revealed "carcinoma *in situ*" in 11. Many *prospective* studies have shown progression of "carcinoma *in situ*" to invasive carcinoma, often after a lapse of many years (Younge, Hertig and Armstrong, 1949; Gusberg and Moore, 1953); but this progression

has not occurred in every case. Unless regression is explained by total removal of the lesion at biopsy, it seems that we are not necessarily dealing with carcinoma at all but with an epithelium which has climbed some of the steps towards malignancy. The data of Petersen (1955; 1959, personal communication) show the proportion of 127 untreated cases which progressed eventually to invasive carcinoma. The cumulative percentage of cases in which carcinoma had developed increased with each year that passed, at first rapidly, then more slowly, and after 7–8 years the risk had become quite small. By extrapolation of the curve it can be deduced that about 35 per cent of cases of " cervical precancerosis " (diagnosed by Petersen) will sooner or later develop carcinoma of the cervix. " Regression " usually occurred in the first year, and 50 per cent of those with lesions persisting beyond this time developed carcinoma within ten years. Whether early invasive carcinoma was present, but not found, at the time of the first biopsy in some of the cases, it is impossible to decide. We also do not know whether the epithelium which developed into carcinoma looked the same as the piece removed at biopsy. These considerations do not invalidate Petersen's figures from the point of view of clinical significance.

If we accept that in only one-third of the cases will "carcinoma *in situ*" progress to invasion, it is important to find out whether the cases can be sorted beforehand into morphological groups carrying different prognoses. The patients are usually young, and many wish for children. Also, if they are all treated as for carcinoma, some may be left with serious complications of treatment (Latour, Brown and Turnbull, 1957). With this in mind we decided to re-examine our material and correlate as far as possible the cytological and histological appearances with the clinical course.

We have also reviewed all the slides from patients whose cervical lesions have been left untreated for a year or more. It seems very important to know whether the abnormal cell-type usually remains the same over long periods, or whether it alters to a more anaplastic type ; and if this happens, whether it happens suddenly or gradually. Data on this point are extremely scarce (Ayre, 1952*a*, *b*) and our series provides evidence about the stability of the cell-type in ten cases.

PRESENT INVESTIGATION

Between 1952 and 1959 10,000 cervical smears were examined from 8522 cases, most of which were gynaecological outpatients. The smears were taken by the examining gynaecologist by scraping the entire squamo-columnar junction area with a wooden spatula as described by Ayre (1952*a*), and smearing the material on a slide, and one slide was made on each occasion. The slides were fixed in ether-alcohol or 3 per cent acetic acid in ethyl alcohol, and they were stained by the Papanicolaou method.

The histological material available has usually been a ring or multiple biopsy of the squamo-columnar region of the cervix. Sometimes a cone biopsy, the amputated cervix or the whole uterus has been submitted. Whenever possible all the cervical tissue available has been embedded and either serial sections or multiple step sections examined.

During the period of 7 years, 11 cases of clinically unsuspected invasive carcinoma of the cervix were detected, as well as 67 cases with possibly precancerous cervical abnormalities. Of the latter group, 56 were clinically unsuspected.

In the same period there were 9 adequate smears which were "false negative" for cervical carcinoma, and 14 "false positive" in which histological studies revealed no cause for the abnormal cells. In some of these cases, it is possible that the area from which the abnormal cells were derived has not yet been removed for examination.

By reviewing the cytological and histological slides from all these cases we hoped to answer the following questions :

1. Can the histological findings be predicted from the cytological appearances?
2. If invasive carcinoma develops from any of the other lesions, can this be predicted either histologically or cytologically? Which types, if any, regress and which persist unchanged?
3. Is the cytological type consistent over a period of time, or does it change either gradually or suddenly?

CLASSIFICATION AND TERMINOLOGY

A system of numerical histological and cytological grading was devised in order to avoid any built-in opinion about the significance of the finding or its prognosis. All the histological slides were reviewed and graded by the same observer (R. H. C.) without knowledge of the clinical or cytological findings. The cytological preparations were independently reviewed and graded by two observers (M. M. B. and A. I. S.), and for this purpose the identification marks of the slides were covered.

A. *Histological Classification*

For the purpose of this investigation we have reviewed all the relevant slides and classified them according to the following numerical system.

Non-invasive lesions

1. Simple basal cell hyperplasia, occurring beneath either squamous or endocervical columnar epithelium (Fig. 1).

2. A thick stratified squamous epithelium with hyperkeratosis. Many of the superficial squames are nucleated and there is variation in size of these nuclei, some being quite large, but the general epithelial pattern is orderly and mitoses are few. The distinction from simple hyperkeratosis is based on the somewhat verrucous variation in the width of the epithelium and the variable size of nuclei in keratinised cells (Fig. 2).

3. Similar to 2 with equally well-defined stratification but more mitotic activity and nuclear irregularity (Fig. 3).

4. Epithelial activity at level of 3 but without the marked hyperkeratosis (Fig. 4).

5. Stratified squamous epithelium, often with considerable variation from normal in the thickness of the strata but with all represented. Mitotic activity greater then 4 and moderate numbers of cells in all layers with large hyperchromatic nuclei (Fig. 5).

6. Epithelium showing some stratification, but poorly developed compared with a normal squamous epithelium. Mitoses and abnormal cells numerous. A thin keratinised layer present on the surface (Fig. 6).

7. Stratification virtually absent but still slight superficial keratinisation in some cases. Many mitoses and abnormal cells (Fig. 7).

Invasive carcinoma

8. Squamous carcinoma of any grade of differentiation in which the tumour cells tend to lie in irregularly shaped solid aggregates with increasing maturity towards the centre, where keratinisation may be present (Fig. 8).

9. Squamous carcinoma including sheets of strikingly uniform large polygonal cells, similar to prickle cells although usually without intercellular bridges (Fig. 9).

10. Squamous carcinoma with a high proportion of grossly abnormal large cells including giant cells with massive irregular nuclei and numerous mitoses, many of them atypical. These tumours present an appearance similar to that seen in a squamous carcinoma soon after irradiation, but the biopsies given this classification were taken before radiotherapy (Fig. 10).

11. A " transitional " type of tumour with broad bands of cells rather than clumps. The general appearance is of fairly uniform hyperchromatism with no keratinisation, and the individual bands of tumour cells are somewhat reminiscent of the epithelium of a " carcinoma *in situ* " (Fig. 11).

Some explanation is required for the choice of the histological classification. As regards the non-invasive lesions, so many names have been applied by different authors that is has become the fashion to quote a sample of them, discard them and start afresh. Hinselmann (1953), in explaining the reason for formulating his rubrics (Hinselmann, 1928), said that in his grading he consciously avoided controversial terms such as pre-cancerosis and pre-invasive carcinoma, only aiming at a more accurate definition of the existing epithelial atypism. Our motives are similar to his, and the present numerical classification was worked out as an experiment in the course of reviewing the present series. The intention was to separate different histological pictures which might give rise to the different cell types found in smears.

Several authors divide epithelial dysplasia of the cervix uteri into two categories, basal cell hyperplasia and carcinoma *in situ*. In basal cell hyperplasia, a variable proportion of the width of the epithelium consists of basal cells and the severity of the lesion is defined in terms of this proportion (Nesbitt, 1955). We have seen very few examples of this straightforward proliferation. Those found are collected as grade 1.

The verbal criteria for diagnosis of "carcinoma *in situ*" are much better defined than the lesions themselves, and it is frequently reiterated that loss of normal stratification is a constant and essential change (Galvin and Te Linde, 1949). In our opinion, much depends on the word " normal ". Bowen's disease of the skin has a range from complete disorganisation of the epidermal pattern to the presence of abnormal cells scattered in all layers in an otherwise fairly well-organised epithelium. Equally, in the cell nests of an invasive carcinoma there is often stratification from cells of basal type at the margin to keratinised squames at the centre. The present classification indicates *inter alia* the degree to which stratification is maintained and this, like the extent of cellular pleomorphism and mitotic activity, appears to vary in a very gradual manner with no sudden steps from one grade to the next. This is also widely recognised (Foulds, 1958). It is a common custom to describe and illustrate one's criteria with great care, the illustrations

covering a single microscopic field, while in practice a diagnosis is reached by examination of the whole of the material available. As regards illustration, the same criticism is inevitable in this series, and it is recognised that the changes seen may vary widely in the same specimen. In general, but not necessarily in detail, the changes in the non-invasive lesions are towards progressively greater " anaplasia " with increasing number, and the number given in each case is the highest applicable. We believe that all workers in this field would regard our grades 6 and 7 as " carcinoma *in situ* ", that many would include grade 5, and that a few would also include grade 4.

The grouping of invasive carcinomata used here has nothing to do with degree of anaplasia as such, but with predominant cell type and arrangement which it was thought might give rise to variations in cell pattern in cervical smears. Grade 8 includes tumours of Broder's Grade 1–4, provided that they conform to the criteria mentioned. The tumours in grade 11 have a pattern much closer to that of " carcinoma *in situ* " than to typical squamous carcinoma.

B. *Cytological Classification*

The predominant abnormal cells seen in smears were, on review, given a numerical classification. This has been done only for the purpose of the present investigation and, like the histological grading, is not intended as a new terminology.

0. Insufficient evidence for suspicion of malignancy.

A–F. In all these grades cells were seen with nuclei showing hyperchromasia and some or all of the other aberrations which are found in carcinoma (namely enlargement, multiplicity, folding or wrinkling of nuclear membrane, prominent nucleoli, aggregation of chromatin into numerous evenly or irregularly distributed granules (chromocentres)).

A. Cells have profuse cytoplasm and are polygonal in outline (Fig. 12).

B. Rounded cells with regular cytoplasm and distinct cell borders. Few small forms with high nucleo-cytoplasmic ratio. (Bi- and multinucleation is common in this type) (Fig. 13).

C. As B, but with predominance of cells showing high nucleo-cytoplasmic ratio and/or small irregularly shaped forms (Fig. 14).

D. Nuclei very variable in shape and size, sometimes pyknotic. Cytoplasm often highly keratinised. Bizarre shapes (tadpoles, fibres) (Fig. 15).

E. Large nuclei, high nucleo-cytoplasmic ratio. Cytoplasm shows little or no keratinisation, stains poorly, and is often indistinctly outlined, torn or lost. Some aggregates may be present (Fig. 16).

F. Nuclei rather uniform and crowded, occurring mainly in aggregates. Separate cells have torn cytoplasm, or are necrotic (Fig. 17).

The cytological grades have been chosen with the idea of making broad morphological distinctions not between isolated cells, but between cell-populations in different smears. Only the abnormal cells under consideration have been graded, and no account has been taken of whether they are many or few, or of the associated epithelial or inflammatory cells.

Our numerical grades are not devised to correspond with any previously published system, but previous authors have used similar categories although with variations in nomenclature.

Papanicolaou (1954) divides the cells desquamating from cervical carcinoma into two main types. Firstly, those of " early malignancy " show nuclear changes but a more or less normal cytoplasm (our grades A, B, C). These " dyskaryotic " cells may be of superficial, navicular or intermediate, parabasal, or endocervical type. Dyskaryosis is shown in cases of intra-epithelial carcinoma or in cases of early invasion, but the superficial and intermediate type may only reflect epithelial dysplasia, and are more likely to regress spontaneously. Secondly, the cells of " advanced malignancy " show cytoplasmic as well as nuclear changes, especially elongation ; they also often occur in large clusters.

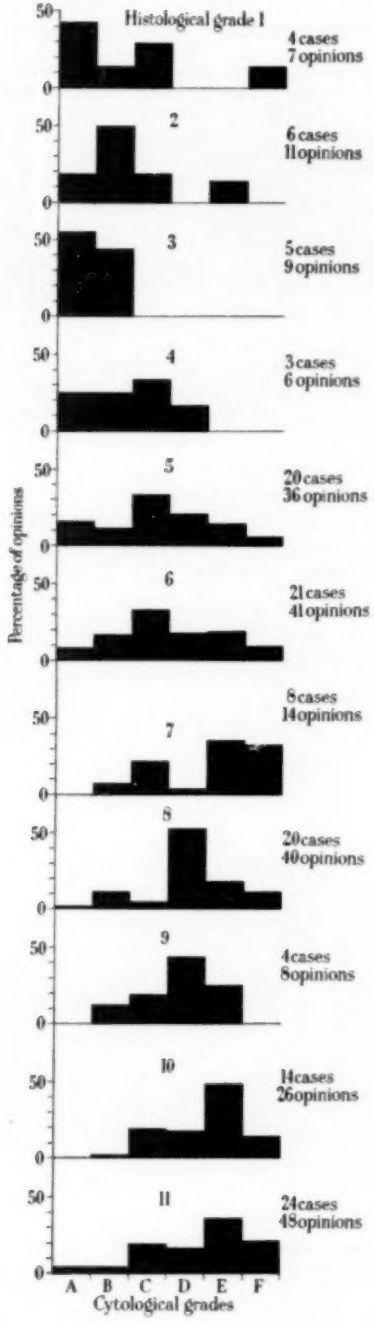
Graham and her co-workers (Vincent Memorial Laboratory, 1950) have given a rather different classification, into " undifferentiated cells " whose cytoplasmic borders are indistinct, and " differentiated " malignant cells with distinct cytoplasmic borders. The second group includes " fibre cells " and "tadpole cells " which Papanicolaou would classify as cells of advanced malignancy, and " third type differentiated cells " with a high nucleo-cytoplasmic ratio, which Papanicolaou would include under " dyskaryosis ", and come into our grade C. Graham's classification is followed by Zinser (1957).

For Graham (1957) the distinction between third type differentiated and non-malignant dyskaryotic cells is one of actual measurement, the malignant cells having a nucleus whose maximum diameter is larger than its distance from the cell border.

Ayre (1952) describes a " precancer-cell-complex " which corresponds roughly to Papanicolaou's " dyskaryosis " and our grades A, B and C, although some " dyskaryotic " cells would appear to fall into Ayre's group of well differentiated cancer cells. Cases of intra-epithelial carcinoma either show " precancer cell complex " or " cancer cells of pre-invasive type ".

It has been maintained (Nieburgs and Pund, 1950 ; Scapier, Day and Durfee, 1952 ; Reagan, Hamonic and Wentz, 1957) that a distinction between carcinoma *in situ* and invasive carcinoma can be made from the cytological appearances. Reagan and his collaborators have made numerous measurements of cervical cells and report that invasion is associated with a change from rounded to elongated or caudate forms, and that this feature is particularly noticeable in the keratinising growths induced by carcinogens in the cervix of the mouse (Reagan and Wentz, 1959). The differences between the smear patterns of carcinoma *in situ* and invasive cancer have been codified by Wied (1956), but the presence or absence of blood, pus and Döderlein's bacilli are considered as well as purely cytological characters, and there must be few who feel any great confidence in making such a forecast. Mackenzie (1955) analysing the cytological appearance in 27 cases of carcinoma *in situ*, found in 15 a predominance of Graham's " third type differentiated " cells, in 9 a picture of bizarre highly differentiated cells exactly as is seen in advanced cancer, and in 3 marked " dyskaryosis " without any cells considered malignant.

McCormack, Belovich and Krieger (1957) have classified the cells found in invasive carcinoma, and describe three main groups, the first two of which correspond to Martzloff's (1923) terminology. The " spinal-cell " group covers the highly keratinised pleomorphic types ; the transitional (the largest group), shows an admixture of large and small forms ; and the small-cell group corresponds to the most anaplastic histological type. Carcinoma *in situ* could not be distinguished with any certainty from invasive cancer.



CYTO-HISTOLOGICAL CORRELATION

For every observation a histological slide was graded as well as the smear taken shortly before. If the two cytological observers gave different gradings, each was entered as a half. More than one entry by the same observer was similarly fractionated. A separate histogram has been made for each histological grade, showing the distribution of cytological assessments found in that grade.

The numbers in each cytological grade are expressed as percentages of the total number for that histogram, so that every histogram totals 100. (The absolute number involved is given on the right.)

Where several observations were made on the same patient, they are treated independently for this purpose.

Histological grade 0 has been omitted. The cases were deliberately selected for cytological abnormality, and obviously the figures cannot be used to show the distribution of cytological findings in cases with normal cervical histology. It is likely that these were biopsies in which the relevant area was missed.

The following deductions can be made from the histograms :

(1) Most histological grades have a corresponding modal cytological grade, but there is so much variation from this that it can never be justifiable to deduce the histology from smears. Even the most sinister-looking cells can be shed from areas of midly unstable epithelium.

(2) On the average, the histological range from slight instability to anaplastic carcinoma is matched by a cytological range, but some types are more characteristic than others. For instance, cytological grades A and B were characteristic of histological grades 1, 2 and 3, and were very seldom found in cases of invasive carcinoma. On the other hand histological grade 7, without invasion, was associated with a very similar distribution of cytological grades to that shown by the most anaplastic invasive carcinomata (Grade 11). Moreover, well-differentiated squamous carcinomata (Grade 8) were matched on the whole by lower cytological

gradings than were the more anaplastic-looking examples of "carcinoma *in situ*" (Grade 7).

It would be a pity to give the impression that the cytological appearances are of no interest beyond indicating the need for a biopsy. They certainly reflect cellular changes which are of biological importance. They do not, however, give any clue to the invasive properties of the deeper parts of the epithelium, of which they represent only the superficial layer.

OBSERVATIONS ON CASES WITH EPITHELIAL INSTABILITY OF THE CERVIX

Sixty-seven cases were seen in which the cervix showed an abnormality short of invasive carcinoma, but classifiable in the histological grades 1–7 described above. Because we do not know where to draw the line between "carcinoma *in situ*" and lesser abnormalities, they will all be included under the heading of "epithelial instability".

In three cases an invasive carcinoma was found at least a year after the first smear. One of these (Case 5) is included although there was no histological evidence of epithelial instability, but grade E cells were present in smears taken two years before the carcinoma was discovered.

There were no deaths or known recurrences following treatment.

In the majority of cases where smears were taken after the biopsy, and before any other surgical procedure, the abnormal cells were found to have disappeared. This was observed in 15 cases, and these have continued to have negative smears. This group represents *either* spontaneous regression *or* complete removal of the

---

EXPLANATION OF PLATES

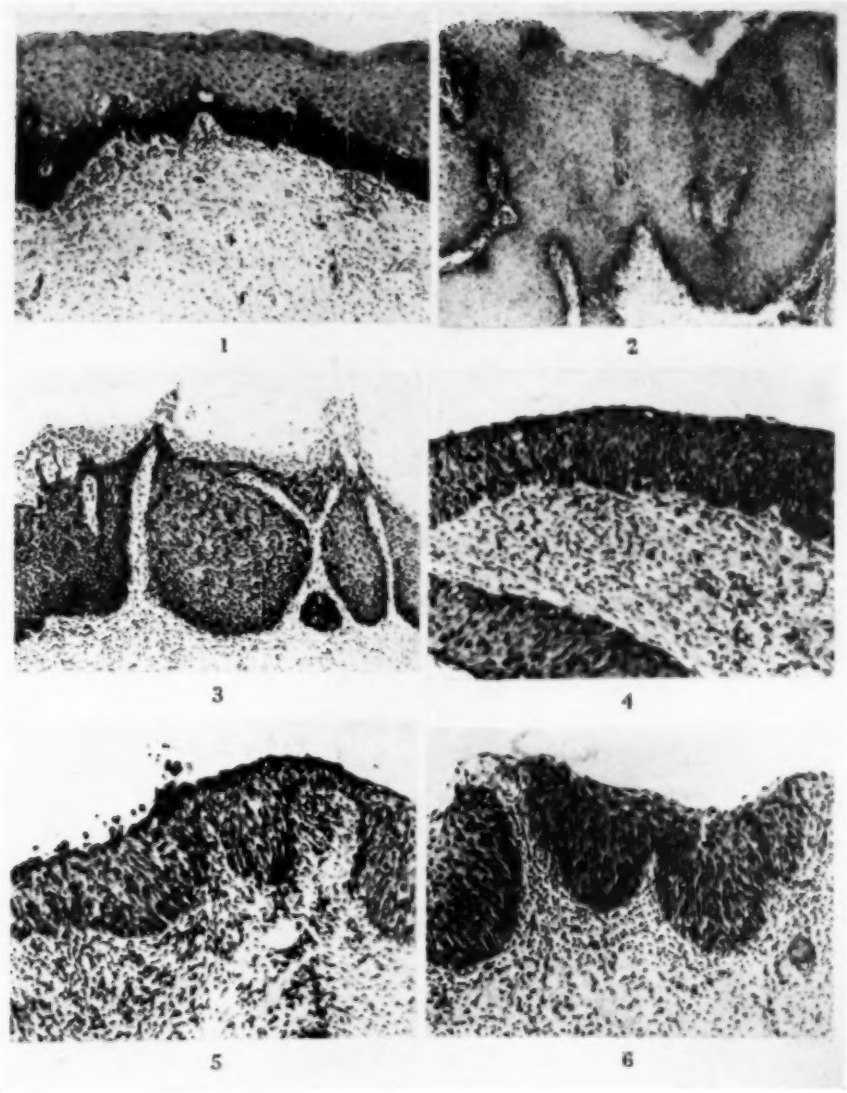
FIGS. 1–11.—Sections of cervical epithelium representative of the gradings used in this study. (H. and E.)

FIG. 1.—Grade 1. ×110.
FIG. 2.—Grade 2. ×40.
FIG. 3.—Grade 3. ×80.
FIG. 4.—Grade 4. ×110.
FIG. 5.—Grade 5. ×110.
FIG. 6.—Grade 6. ×110.
FIG. 7.—Grade 7. ×110.
FIG. 8.—Grade 8. ×55.
FIG. 9.—Grade 9. ×110.
FIG. 10.—Grade 10. ×55.
FIG. 11.—Grade 11. ×55.

FIGS. 12–17.—Papanicolaou-stained cervical smears showing cells representative of the gradings used in this study. ×350.

FIG. 12.—Grade A.
FIG. 13.—Grade B.
FIG. 14.—Grade C.
FIG. 15.—Grade D.
FIG. 16.—Grade E.
FIG. 17.—Grade F.

FIG. 18.—Case 2, first smear. ×350.
FIG. 19.—Case 2, smear taken three years later. ×350.
FIG. 20.—Case 4, first smear. ×350.
FIG. 21.—Case 4, smear taken a year later. ×350.
FIG. 22.—Case 5, first smear. ×350.
FIG. 23.—Case 5, smear taken two years later. ×350.
FIG. 24.—Case 6, first smear. ×350.
FIG. 25.—Case, 6, smear taken almost three years later. ×350.
FIG. 26.—Case 9, first smear. ×350.
FIG. 27.—Case 9, smear taken six months after the first. ×350.
FIG. 28.—Case 9, smear taken fifteen months after the first. ×350.

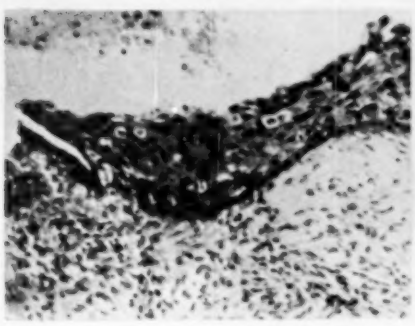


1 2

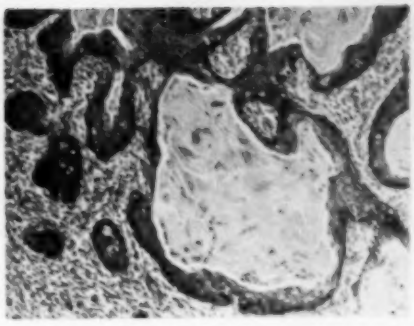
3 4

5 6

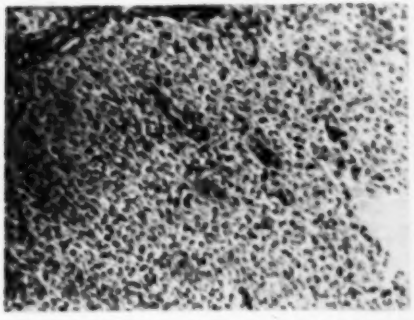
Boddington, Cowdell and Spriggs.



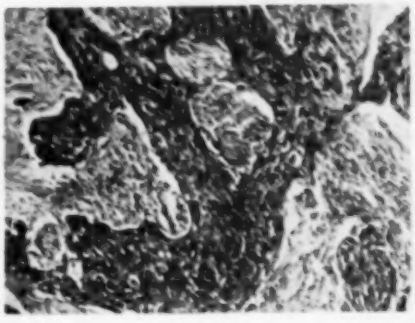
7



8



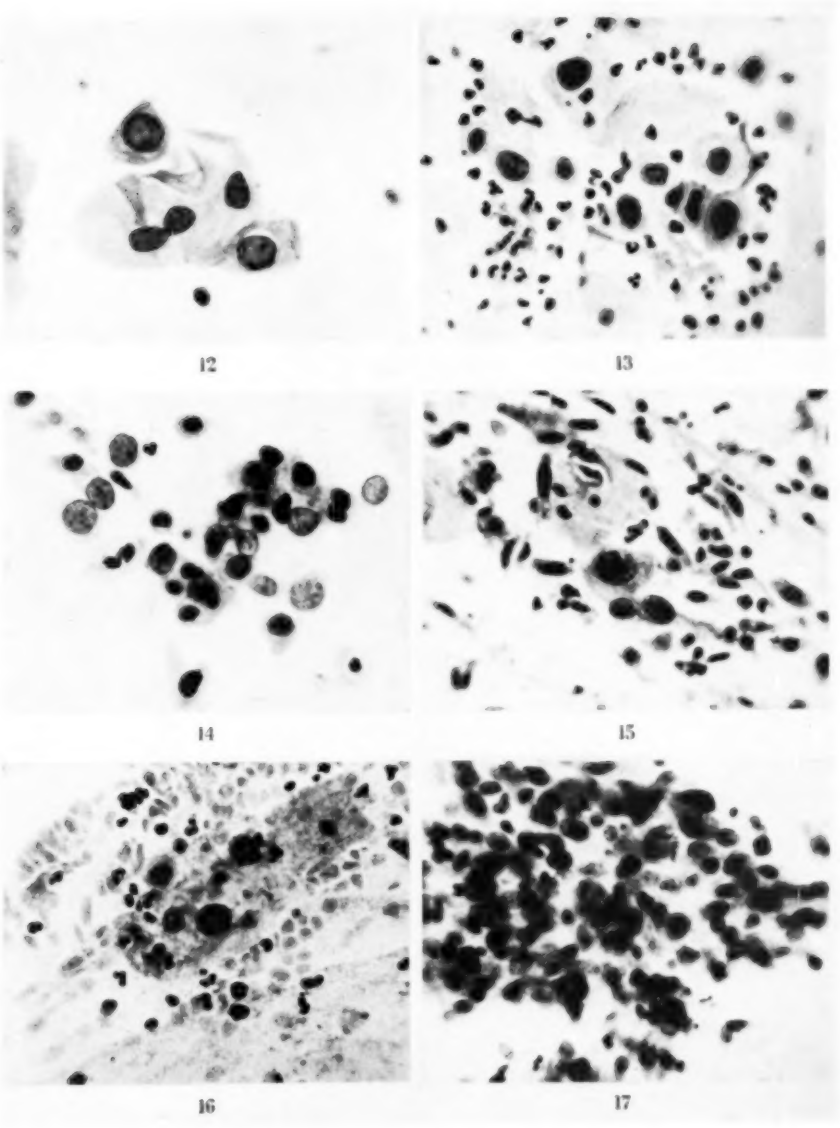
9



10

11

Boddington, Cowdell and Spriggs.



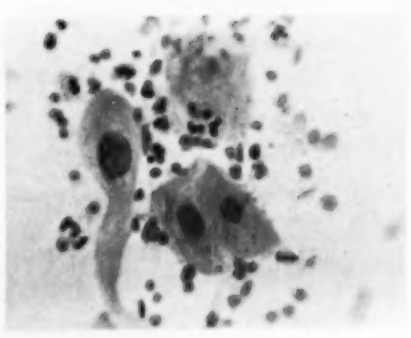
12 13

14 15

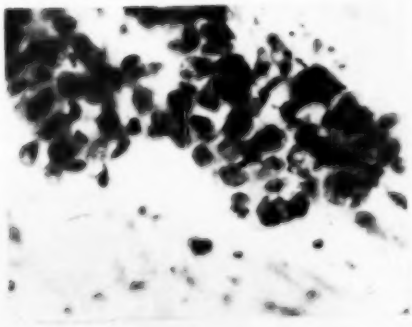
16 17

Boddington, Cowdell and Spriggs.

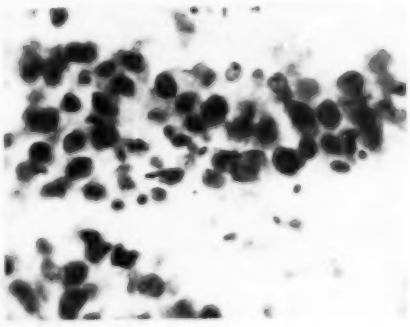
18



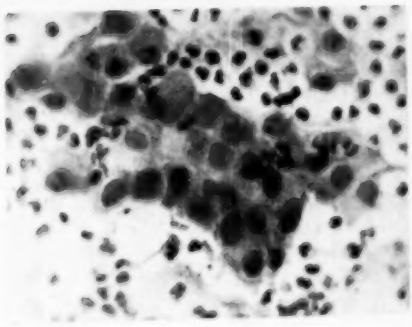
19



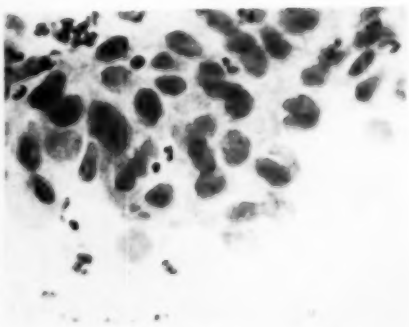
20



21

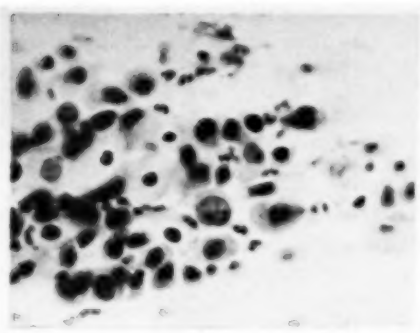


22

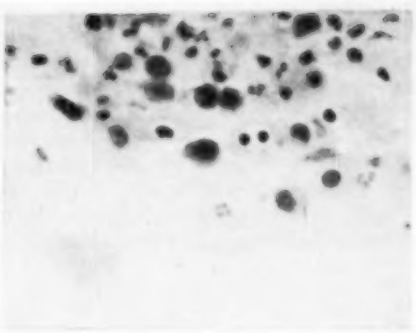


23

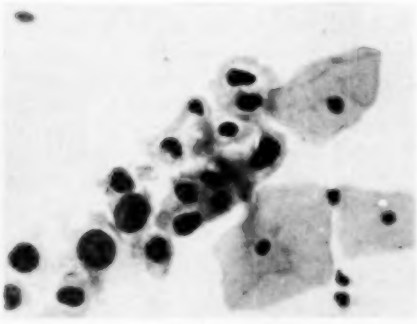
Boddington, Cowdell and Spriggs.



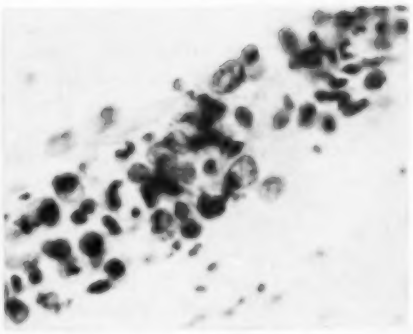
24



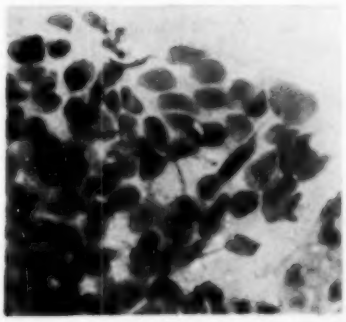
25



26



27



28

Boddington, Cowdell and Spriggs.

lesion at biopsy. In three other cases the smears were still positive after the first biopsy, but became negative after subsequent ones.

Usually the course of the disease was interrupted by surgical treatment, and nothing more can be learnt from these cases. There was, however, a group of ten cases in which, for various reasons, a persisting lesion was followed by repeated smears for at least a year. In four of these the period of observation was 2–3 years, and in one was 5 years. These are a selected group, but are of particular interest because the literature contains very few details about the changes, if any, to be found in the desquamated cells over periods of time. Is there a continuous transformation towards more anaplastic types? Or are such transformations sudden, punctuating long periods of morphological stability? Or does the cell-type remain unchanged, even if invasion supervenes? The following brief description accompanied by photographs show the different patterns of cellular behaviour observed in this series.

A. *Persistence of abnormal cells without cytological progression**

Case 1 (Reg. No. 35470) aged 33.—Complaint—irregular excessive bleeding. Six smears were taken over a period of $3\frac{1}{2}$ years. The first four showed cytological grades A + C, but two taken in the last year showed little abnormality. Three biopsies all showed grade 6 lesions. Hysterectomy was finally performed, and sections of the cervix showed areas of grade 6 and of more extensive grades 3 and 1.

Case 2 (Reg. No. 34924) aged 49.—Complaint—irregular periods. The cervix was eroded. The smear showed grade A cells (Fig. 18). She failed to attend for biopsy and was next seen 3 years later. A further smear still showed grade A cells (Fig. 19) and a biopsy showed a grade 3 lesion. She is still being followed.

Case 3 (Reg. No. 29734) aged 38.—Complaint—irregular prolonged periods. Normal looking cervix. Cytological grade C. A biopsy showed a grade 4 lesion. She was not seen for 5 years, after which another smear showed abnormal cells but now of grade B. A recent biopsy shows no abnormality.

Case 4 (Reg. No. 29653) aged 33. Complaint—vaginal discharge. A small erosion was present. Smears graded E–F (Fig. 20), biopsy grade 6. The same cell type was repeatedly found in smears taken over a period of a year (Fig. 21) and a second biopsy 10 months after the first was again grade 6. Hysterectomy was then performed, and no atypical epithelium remained in the specimen.

Case 5 (Reg. No. 33994) aged 35.—First seen in early pregnancy with a complaint of bloodstained discharge. There was a vascular erosion. Smears showed grade E cells (Fig. 22). Biopsy was not done at this time, and two smears taken later in pregnancy were reported as negative, but on review one of them shows similar cells. Two years later similar abnormal cells were still present (Fig. 23), and a biopsy revealed an early carcinoma (grade 8). A Wertheim's hysterectomy was then performed.

Case 6 (Reg. No. 21090) aged 33.—Complaint of vaginal discharge. The cervix was eroded. Smears showed grade B–C cells (Fig. 24), biopsy grade 1. She was followed with repeated cervical smears for nearly three years, during which time she had two pregnancies (one ending in abortion, the other ectopic). The same type of cell was always present, though fluctuating in numbers (Fig. 25). Thirty-

* "Cytological progression" means alteration of the abnormal cell type to a higher grade, whether or not the lesions became invasive.

four months after the first smear, a biopsy showed a very small carcinoma (grade 8).

Case 7 (Reg. No. 32352) aged 37.—Complaint—dyspareunia. Smears showed grade C cells, and biopsy showed a grade 1 cervical epithelium. During the following year two further smears were taken, without appreciable change, and two further biopsies showed no atypical epithelium. Unfortunately no further smears were obtained during the following year, but a hysterectomy was performed two years after the first smear on account of menorrhagia and pelvic pain, and the cervix contained a very small grade 8 carcinoma.

The series of smears is incomplete in this case, but the original cell type persisted for at least a year.

Case 8 (Reg. No. CG/3475) aged 29.—At post-natal examination a large cervical erosion was found, and smears showed grade E–F cells. A biopsy showed a grade 7 lesion. As the smears continued positive for 2 months without change of cell type, the cervix was amputated. The specimen could not be graded owing to previous cauterisation. Subsequent smears still showed abnormal cells, but these were of a different sort (grade B). Another biopsy 6 months after the amputation showed no abnormality, but a third after a further 6 months showed a grade 5 lesion. The cellular picture remained unchanged, and a total hysterectomy was performed, retaining the ovaries.

After this the smears from the vaginal vault have remained abnormal (grade A–B). A biopsy taken from the vault 5 years after the first shows an unstable vaginal epithelium (grade 5).

B. *Progression of cell type during observation*

Case 9 (Reg. No. 33443) aged 28.—Complaint of infertility. Normal cervix. Smear showed cells of grade C (Fig. 26). Biopsy showed a grade 5 lesion. A series of 15 smears were taken over a period of 15 months, during which a normal pregnancy and delivery took place. Six months after the first smear the abnormal cells began to become more crowded, with a reduction in their cytoplasm (Fig. 27). The last smear of the series, taken 3 months post-partum, showed abundant grade F cells (Fig. 28) and cervical amputation was performed. The sections showed a grade 7 lesion.

C. *Regression after persistence for a year*

Case 10 (Reg. No. 29506) aged 27.—Complaint—dysmenorrhoea. There was a third degree tear, and she was admitted for repair. At this time a cervical smear showed grade C–E cells. Repeated smears showed the same type of cells, and a biopsy 8 months after the first smear showed a grade 5 lesion. The abnormal cells persisted for a further 4 months, when a second biopsy was done. This showed only chronic cervicitis, and all subsequent smears were negative during an observation period of 4 years. Two further biopsies showed chronic cervicitis only.

This series of cases illustrates three points.

1. The same cytological type often persists with only slight fluctuations over considerable periods of time, and does not show continuous progression towards more anaplastic types.

2. Invasion can almost certainly supervene in areas of epithelial instability, although it is impossible to prove that the lesion was not invasive at the outset.

In three of the above cases invasive carcinoma was eventually found after at least a year's observation (Cases 5, 6, and 7) and in none of them was any cytological progression observed.

3. In case 9 the abnormal cells showed an unmistakable cytological progression. The smears were not all comparable and it is difficult to be sure whether to record a definite change at 14 weeks pregnancy, but the final smear taken 3 months after delivery showed entirely new appearances, with marked crowding of nuclei and loss of distinct cytoplasmic borders. There was also a histological change from grade 5 to grade 7. This example suggests that morphological alterations, when they occur, proceed stepwise rather than by a continuous transformation. Ayre (1952*b*), in a case studied with over 200 smears, found a " slow progression " until the rather sudden disappearance of the abnormal cells. In his book (Ayre, 1952*a*) changes in cell type are illustrated in " Cell Behaviour Studies " cases 1 and 3, but it is not clear whether the alterations were sudden or gradual ; case 3 showed one distinct change, then long persistence of the same type. Many more cases are required to settle this point.

DISCUSSION AND CONCLUSIONS

Because of the accessibility of the cervix uteri for smears and biopsies, more is known about the earliest stages leading to cancer at this site than at any other. We are able to recognise lesions which carry a measureable risk (about 30–40 per cent) of sooner or later becoming malignant (Petersen, 1955). As this development probably occurs in a random fashion, it does not seem to be possible to predict the course in any particular case.

Unfortunately, the picture has been confused by disputes about whether this or that histological appearance should be called carcinoma or not. Except when there is evidence of invasion, the question is one of words and not of facts. It is unfortunate that the terms " carcinoma *in situ* ", " intra-epithelial carcinoma " and " stage 0 carcinoma " have been introduced, as they are most difficult to define and no two pathologists will agree about their exact use.

We recommend the term " epithelial instability " to cover all the histological lesions illustrated in Fig. 1–7. Basal-cell hyperplasia (our grade 1) might seem out of place here, but since in two cases (cases 6 and 7) it preceded invasive carcinoma by 2–3 years, we feel that it should be treated with the same respect as the other grades which we have illustrated.

In any case found to have epithelial instability of the cervix it is of the utmost importance to discover whether there is in fact an early invasive carcinoma which has been missed. Several authorities have suggested that a distinction can be made fairly reliably from cytological evidence. We cannot confirm this. Our cytological gradings, while roughly reflecting the cell-types seen in the corresponding histological sections, have not been correlated with the presence or absence of invasion, and we think it wrong to make guesses on the basis of cervical smears. Even the biopsy sometimes fails to reveal an invasive carcinoma ; a full histological study of a ring biopsy of the squamo-columnar junction should be made in every case where this problem arises.

In the present series, the majority of untreated cases ceased to have positive cervical smears after one or more biopsies, and the abnormality has not recurred. It is impossible to determine whether these lesions regressed spontaneously or

whether they were completely removed by the biopsy ; most probably there have been examples of each. In ten cases a persistent lesion was followed for over a year, and three of these were eventually found to have small invasive carcinomata. Unfortunately our figures cannot be used to assess the probability of development of carcinoma ; selection was exercised at all stages, and the cases with persistent lesions which remained untreated are only a small residue of the whole series ; also the follow-up period is very short. Our experience has however, been quite consistent with the pattern shown in Petersen's (1955) series ; he found that in approximately 50 per cent of cases with " cervical precancerosis " the lesion disappeared within a year, but that if it persisted, carcinoma developed within 8 years in 50 per cent (13 out of 26).

Are the lesions which regress different histologically or cytologically from those which persist? The analysis of our findings shows no morphological criterion correlated with persistence or regression, nor with the subsequent development of carcinoma. In practice the distinction between persistent and transient lesions is easily made by the use of repeated cervical smears. In the present series those smears which became negative nearly always did so following the first biopsy ; case 10 is the only striking exception.

The series of persistent lesions provides some interesting evidence for the " stepwise " progression of premalignant states. As the paired illustrations show, the abnormal superficial cells shed from areas of epithelial instability are not steadily changing towards a more anaplastic appearance. In the relatively short periods over which we have been able to observe them they have usually remained true to type, just as malignant cells generally do both in experimental conditions and in human disease. In case 9 a sudden change was observed, consistent with the development of a new cell clone. The discovery of invasive carcinoma in two cases while under repeated observation was *not* associated with any alteration in cell type. Evidently, it is unjustifiable to expect any cytological warning before invasion supervenes.

The problem confronting the gynaecologist in these cases is never twice the same, and no generalisations can be made about the appropriate treatment of " epithelial instability ". Above all, no help can be expected from the study of the abnormal smears or biopsies in deciding the prognosis of an individual case. There is no urgency, and provided that the patient is kept under surveillance there does not appear to be any danger. On the other hand, a lesion of this type which does not disappear after biopsy is undoubtedly " precancerous ", in the sense that cancer is sooner or later very likely to develop, and the cervix or the uterus ought to be removed, and negative smears obtained, before the patient is discharged from observation.

SUMMARY

Now that 10,000 cervical smears have been examined from gynaecological outpatients in Oxford, cytological and histological material pertaining to carcinoma of the cervix and possible precancerous lesions has been reviewed.

The lesions found at biopsy have been given numerical gradings. The different types of abnormal cells seen in the smears have also been classified numerically, and a comparison has been made between the cytological and the histological gradings. Although there is a rough correlation between certain cytological and

histological types, the presence or absence of invasion is not correlated with distinct cytological patterns.

Sixty-seven cases were found of possibly precancerous lesions (including " carcinoma *in situ* "). In 33 of these the cervix or uterus was removed surgically without any further observations. In 18 the lesion could no longer be detected after biopsy, having either regressed or been removed entirely by the biopsy. Cervical smears were persistently positive for a year or more in 10 cases, and three of these eventually had invasive carcinoma.

Review of the series of smears from these persistently positive cases showed that the abnormal cell type remained remarkably constant throughout, even over a period of several years. There was one exception, where a sudden progression occurred to a more anaplastic-looking type. In the cases where invasive carcinoma supervened, no alteration in cell type was observed.

The data from this series are consistent with the idea that epithelial instability (basal cell hyperplasia, " carcinoma *in situ* " and related lesions) usually disappears without any treatment more extensive than biopsy, but sometimes becomes irreversible, and in that case sooner or later develops without any cytological warning into invasive carcinoma.

If the smears and biopsies do not become normal within the first year, the lesion should be regarded as precancerous.

We should like to express our thanks to Professor J. Chassar Moir for giving us the facilities of the Nuffield Department of Obstetrics and Gynaecology during a large part of this work ; and to Mr. J. Stallworthy for his constant support and interest. We are very grateful to Dr. A. H. T. Robb-Smith for his advice and help, and to the British Empire Cancer Campaign, from whom A. I. S. and M. M. B. receive whole-time grants. We thank Mrs. J. Shimmin, Mrs. R. Hughes, Miss C. Clarke and Miss G. Olah for their assistance.

REFERENCES

Ayre, J. E.—(1952*a*) 'Cancer Cytology of the Uterus'. London (Churchill).—(1952*b*) *Sth. med. J., Nashville*, **45**, 915.
Babès, A.—(1928) *Pr. méd.*, **36**, 451.
Carson, R. P. and Gall, E. A.—(1954) *Amer. J. Path.*, **30**, 15.
Foulds, L.—(1958) *J. chron. Dis.*, **8**, 2.
Galvin, G. A., and TeLinde, R. W.—(1949) *Amer. J. Obstet. Gynec.*, **57**, 15.
Idem, Jones, H. W. and TeLinde, R. W. (1952) *J. Amer. med. Ass.*, **149**, 744.
Graham, R.—(1957) *Acta Cytol.*, **1**, 23.
Gusberg, S. B. and Moore, D. B.—(1953) *Obstet. Gynec.*, **2**, 1.
von Haam, E. and Scarpelli, D. G.—(1955) *Cancer Res.*, **15**, 449.
Hinselmann, H.—(1928) *Zbl. Gynäk.*, **52**, 2797.—(1953) *Z. Geburtsh. Gynäk.*, **138**, 153.
Howard, L., Erickson, C. C. and Stoddard, L. D.—(1951) *Cancer*, **4**, 1210.
Latour, J. P. A., Brown, L. B. and Turnbull, L. A.—(1957) *Amer. J. Obstet. Gynec.*, **74**, 354.
McCormack, L. J., Belovich, D. and Krieger, J. S.—(1957) *Amer. J. clin. Path.*, **28**, 179.
Mackenzie, L. L.—(1955) *Amer. J. Obstet. Gynec.*, **69**, 629.
Martzloff, K. H.—(1923) *Johns Hopk. Hosp. Bull.*, **34**, 141 and 184.
Nesbitt, R. E. L.—(1955) *Obstet Gynec.*, **6**, 239.
Nieburgs, H. E. and Pund, E. R.—(1950) *J. Amer. med. Ass.*, **142**, 221.

PAPANICOLAOU, G. N.—(1928) *Proc. 3rd Race Betterment Conf.*, 528.—(1954) 'Atlas of Exfoliative Cytology'. Cambridge, Mass. (Commonwealth Fund.)
PETERSEN, O.—(1955) 'Precancerous Changes of the Cervical Epithelium'. Copenhagen (Danish Science Press).
REAGAN, J. W., HAMONIC, M. J. AND WENTZ, W. B.—(1957) *Lab. Invest.*, **6**, 241.
Idem AND WENTZ, W. B.—(1959) *A.M.A. Arch. Path.*, **67**, 287.
SCAPIER, J., DAY, E. AND DURFEE, G. R.—(1952) *Cancer*, **5**, 315.
VINCENT MEMORIAL LABORATORY, STAFF OF—(1950) 'The Cytologic Diagnosis of Cancer'. Philadelphia (Saunders).
WIED, G. L.—(1956) *Amer. J. Obstet. Gynec.*, **71**, 793.
YOUNGE, P. A., HERTIG, A. T. AND ARMSTRONG, D.—(1949) *Ibid.*, **58**, 867.
ZINSER, H. K.—(1957) 'Die Zytodiagnostik in der Gynäkologie'. Jena (Fischer).

ADENOCARCINOMA OF THE UTERUS IN INFANCY

A. G. MARTINS

*From the Alder Hey Children's Hospital, Liverpool**

Received for publication March 17, 1960

It is difficult to obtain accurate information about adenocarcinoma of the uterus in infancy : references to it are rare and some reports are lacking in detail.

According to Speert (1947) only about thirty cases of cancer of the cervix had been described in the first two decades of life. In the same paper that author reported the seventh case of adenocarcinoma of the cervix in girls aged 12 or under. He stated that no case with epidermal carcinoma of the cervix had been recorded in that age group.

Boyes, quoted by Hark (1958), reports what he believes to be the fifth case of adenocarcinoma of the cervix in infants under one year of age : an 11-months-old child which presented with a 3 weeks' history of vaginal bleeding and was found at laparotomy to have an inoperable growth. Treated with radiotherapy (Cobalt Bomb) with some improvement, the patient died with diffuse metastases 11 months later.

Amesse (1932), quoted by Jolly (1955), described an adenocarcinoma " involving " the uterus in a girl aged 23 months. Lockhart (1935), quoted by Speer (1947), described a papillary adenocarcinoma of the fundus in a 14-months-old girl.

The fact that adenocarcinoma of the cervix seems more common than adenocarcinoma of the fundus in children is in keeping with the observation in adults that the relative incidence of carcinoma of the fundus increases with age. It is well known that the cervix constitutes the major part (two-thirds) of the premenarchal uterus and that in mature women that relation is reversed.

Speert (1947) postulates a theory to explain the non-occurrence of epidermal cancer of the cervix in children. He states that the lack of oestrogen stimulation in this age period maintains the cervical epithelium in relative quiescence (the thickness of the epithelium is only a quarter or a fifth of that in mature women, mitosis are much less frequent and the functional zone between stratified squamous epithelium and columnar epithelium is more stable). Although this is an interesting hypothesis it does not represent the answer and the solution of the problem must be looked for amongst more general biological factors accounting for the failure of epidermal tumours to develop in the young, not only in the cervix but in the skin and other situations.

It is well known that most children's tumours are embryomas. The uterus is no exception and so the commonest tumour to be found in infancy is the so-called " embryonal sarcoma of the urogenital sinus ", also known as rhabdomyosarcoma or sarcoma botryoides, which originates most frequently at the upper end of the vagina and may later invade the uterus. Gross (1956) refers to 7 children with

* Present address : Av. António Augusto Aguiar, 114 R/C., Lisboa-1, Portugal.

this type of tumour treated at the Boston Medical Center but he has not seen a single carcinoma.

The differential diagnosis between adenocarcinoma of the uterus and embryonal sarcoma is important, because the extent of radical surgery can depend on it.

Case Report

H. C—, d/b 26.i.58.—This child having previously been admitted to another hospital for observation and treatment following a recent history of passing blood per urethra or per vagina, was transferred to Alder Hey Children's Hospital on February 9, 1959, when 12 months old. She was a healthy looking infant (Fig. 1), but rectal examination revealed a hard, fixed mass, the size of a golf ball, bulging into the anterior rectal wall, which seemed to be uterine in origin. Intravenous pyelogram as well as X-rays of chest and spine were normal. Cystoscopy showed the urethra and bladder pushed over to the right side, with no evidence of invasion of the bladder wall.

The day after her transfer to Alder Hey, operation was performed through a T-shaped incision (Fig. 2) and the diagnosis of a tumour of the uterus was confirmed. Biopsy and frozen sections showed that the tumour was malignant. The pubic bones were separated at the symphysis, the ureters were divided and the bladder, urethra, uterus, tubes, ovaries and vagina were removed in continuity (Fig. 3). A single enlarged lymph gland was removed from the pelvis ; it contained in its sinuses cells similar to those of the primary tumour. The ureters were anastomosed to the recto-sigmoid junction.

On macroscopic examination the tumour was 4·5 cm. in diameter, firm, almost hard and its cut surface was homogenously white, with no areas of haemorrhage or necrosis. The tumour completely filled the uterine cavity and extended down into the cervical canal.

Microscopy showed a fairly well differentiated mucus-secreting adenocarcinoma with areas of greater anaplasia (Fig. 4), which had penetrated deeply through the wall of the fundus and reached the peritoneal surface. The posterior bladder wall was not penetrated and no invasion of blood vessels was seen.

The child stood the operation well and apart from an intercurrent infection with *B. coli* 055 (infantile gastro-enteritis) had an uneventful post-operative recovery. Post-operative radiotherapy was considered but not favoured. The child was discharged 6 weeks after operation. Rectal examination and blood chemistry performed immediately before discharge were normal. The patient was passing urine per rectum about every 2 hours and was fairly continent.

She was re-admitted 1 month later because of diarrhoea and vomiting, clinical examination was negative and the symptoms promptly subsided. Because her

EXPLANATION OF PLATE

FIG. 1.—Child before operation.

FIG. 2.—Type of incision used.

FIG. 3.—Specimen removed at operation : bladder, urethra, vagina and cervix, which is invaded by tumour throughout.

FIG. 4.—Microphotograph : low power magnification showing the typical appearance of the adenocarcinoma.

1

2

3

4

Martins.

blood urea was slightly raised an intravenous pyelogram was performed, which showed a normal left pyelogram but no evidence of excretion on the right side. A fortnight later the symptoms recurred and a large mass was now felt in the right side of the abdomen ; rectal examination was still negative.

Exploratory laparotomy was performed on June 1, when a large inoperable tumour mass was found filling the right side of the abdomen. A large amount of dark brown mucoid fluid was aspirated from the tumour and a biopsy performed. The biopsy specimens were unfortunately non-significant but the cytology of the aspirated fluid suggested tumour tissue. The wound healed well but the child's condition progressively deteriorated and, apart from general supportive measures, no treatment was considered justifiable. The child died on June 29, 1959. Unfortunately permission for autopsy was not obtained.

DISCUSSION

The tumour in our case extended to the peritoneal surface of the fundus and involved the entire cervix. As often happens in advanced lesions, it is difficult or impossible to ascribe the origin of the tumour to the body of the uterus or to the endocervix and it is therefore preferable for purposes of classification to classify our case as an " adenocarcinoma of the corpus and endocervix ".

The initial symptom (but unfortunately late in the disease) is usually vaginal bleeding in an otherwise healthy child, in whom clinical examination is negative except for the presence of a low pelvic, painless mass. The presentation of embryonal sarcoma of the upper vagina may be similar and clinical distinction impossible unless the characteristic colourless, grape-like, cystic masses can be seen on vaginal examination (clinical or endoscopic).

Endoscopic examination can be performed even in small infants either with a bronchoscope or with the McCarthy cystoscope, using a flow of saline through the instrument to distend the vagina.

Vaginal or cervical cytology can be a help in the diagnosis of malignancy and its type but should not delay treatment.

Treatment

Considering that the very few cases reported have all died, it is difficult to say how they should be treated. By analogy with adults, the following remarks seem justified.

Technical difficulties due to size, etc., would preclude the use of intra-uterine radium, even in cancer limited to the cervix.

Radiotherapy in " curative " doses would be too harmful to pelvic growth, if given in this age group, so that its very doubtful benefits in a relatively radio-resistant growth such as this one are certainly surpassed by the disadvantages.

Radical hysterocolpectomy *en bloc* with pelvic lymph node dissection would seem to be the most adequate form of treatment. Removal of the bladder, urethra and rectum should not be necessary unless they are involved in the malignancy.

In embryonal sarcoma radical surgery is again the treatment of choice, but should probably be more radical than in adenocarcinoma. This tumour tends to recur in organs derived from the uro-genital sinus and so the bladder and urethra

should be removed even if not clinically involved in the tumour (Schakman, 1950), although a more conservative approach, namely simple total hysterocolpectomy, has been used with success (Ulfelder and Quan, 1947 ; Gross, 1956). Metastases are relatively late but kill quickly once they have occurred.

This suggested difference in the extent of radical surgery makes it important that a bopsy should be taken at laparotomy and the result of a frozen section examination obtained. The pathologists may only be able to state that the tumour is malignant (we have been unable to find any reference to benign tumours of the uterus or vagina in children), but he may recognize the typical mesenchymal cells or rhabdomyoblasts of an embryoma or the malignant epithelial cells of an adenocarcinoma.

Unfortunately, so far, results have been disappointing : no survival has as yet been recorded for adenocarcinomas of the uterus in infancy, which are extremely malignant and of much worse prognosis than in adults. So far only one case (Lockhart's (1935) patient, who was treated by radiotherapy and died 17 months later of renal failure due to involvement of the bladder) has lived for more than a year after the diagnosis was made.

Radical operation is performed through a combined abdomino-perineal approach, either with a vertical or a T-shaped skin incision (separation of the pubic bones improving the exposure).

SUMMARY

A further case of " adenocarcinoma of the corpus and endocervix " in a 12-months-old child presenting with a 3 weeks' history of vaginal bleeding is described. The extremely rapid evolution of this lesion in children is emphasized as well as its rarity.

The non-occurrence of epidermoid tumour of the uterus in children is noted.

The treatment advised is early and radical hysterocolpectomy *en bloc* with pelvic lymph node dissection through combined abdominal and perineal approach.

Radiotherapy is not indicated, except for palliation.

I am indebted to Miss Isabella Forshall for allowing me to publish this case, to Drs. Edward Hall and Jean Bouton for their advice in the pathology aspects, to Mr. Rodney Green for the photographs, to Mr. Charles Fitzsimons for the microphotographs and to Mrs. Isabelle Hunter for the typescript.

REFERENCES

Amesse, J. W.—(1932) *Colorado Med.*, **29**, 317.
Gross, R.—(1956) ' The Surgery of Infancy and Childhood ', Philadelphia, (W. B. Saunders & Co.)
Hark, B.—(1958) *Pediat. Clin. N. Amer.*, **5**, 95.
Jolly, H.—(1955) ' Sexual Precocity ', Springfield, Ill. (Ch. C. Thomas).
Lockhart, H. A.—(1935) *Amer. J. Obstet. Gynec.*, **30**, 76.
Schakman, R. (1950) *Brit. J. Surg.*, **38**, 26.
Speert, H.—(1947) *Amer. J. Obstet. Gynec.*, **54**, 982.
Ulfelder, H. and Quan, S. H.—(1947) *Surg. Clin. N. Amer.*, **27**, 1240.

THE ARSENIC CONTENT OF BRONCHIAL MUCOSA AND SUBMUCOSA IN MAN.

A Comparison of Specimens from Lung Cancer Victims and Control Tissue

R. H. HOLLAND, A. R. ACEVEDO, and D. A. CLARK

From the Medical Research Service of the Veterans Hospital, and the Surgery Department, University of Texas Southwestern Medical School, Dallas, Texas

Received for publication March 3, 1960

Populations exposed to arsenic dusts and fumes have a high incidence of lung cancer according to six recent reports by Liebegott (1949), Hess (1956), Lull and Wallach (1956), Osburn (1957), Roth (1957) and Braun (1958). Additional evidence by Satterlee (1956) and Holland *et al.* (1958) has shown a high arsenic concentration in urban atmosphere, and a 200–600 per cent increase in the arsenic content of most American cigarette tobaccos from 1932 to 1957. Thus the arsenic inhaled from our environment, cigarette smoke, etc., becomes a potential carcinogen when deposited in our respiratory systems and is worthy of exhaustive investigation.

There are no known analyses of the arsenic content of bronchial mucosa and submucosa, however, Bailey (1957) and Sula and Zelenkova (1957) did determine the arsenic content of parenchymal lung tissue and bronchial lymph nodes. It is interesting to note that the latter two investigators found 2–70-times more arsenic in the bronchial lymph nodes than in any other organ in the body and the more anthracotic the lung and bronchial lymph nodes the higher was the arsenic content. Unfortunately, these studies did not include bronchial mucosa and submucosa, where most, if not all primary lung cancers arise. Therefore, it was felt that an arsenic analysis of these strata should be performed on autopsy specimens from lung cancer victims and from cadavers that showed no evidence of neoplastic disease in the respiratory system.

MATERIAL FOR ANALYSES

The specimens in this study were removed from male cadavers whose ages, occupations and smoking histories can be found in Table I. The lower trachea, carina, main stem and lobar bronchi were removed intact with their lymph nodes from 15 victims of lung cancer and from 23 cadavers who showed no evidence of neoplastic disease in the respiratory system grossly or on histological examination. The specimens were removed within 10 hours after death and the mucosa with its submucosa was stripped from the underlying fibrous tissue and placed in a Petri dish (Fig. 1). The lymph nodes were then dissected from the specimen and treated in a similar manner.

TECHNIQUE FOR ANALYSES

About 1 g. of each wet specimen (which we found the ideal weight for analysis) was placed on an ordinary previously weighed tissue slide. The wet weight was determined by subtracting the weight of the slide from the weight of wet tissue and the slide. The specimen was then placed in an oxygen bomb and dried for three to four hours at a temperature of from 60 to 80° C. while a continuous stream

TABLE I.—*As_2O_3 Content of Bronchial Mucosa and Submucosa*

A. *Specimens from Lung Cancer Victims*

Case	Age	Occupation	Smoking history	As_2O_3 p.p.m. mucosa and submucosa
C. D—	69	Railroadman	2–3 pkg./day	5·9
J. A. D—	63	Oil man	2–2½ pkg./day	6·5
H. B—	63	Carpenter	2–3 pkg./day	6·4
E. K. S—	62	Plumber	1 pkg./day	6·3
H. D—	64	Painter	1½ pkg./day	6·7
W. L—	46	Laborer	Unknown	2·3
E. J. J—	71	Railroadman	1½–2 pkg./day	13·7
L. H—	55	Presser	3 pkg./day	13·4
C. T. B—	62	Paper hanger	2 pkg./day ; pipe	5·0
J. B. W—	40	Engineer	2 pkg./day	4·5
O. P—	43	Watchmaker	1–1½ pkg./day	1·3
R. W. G—	64	Mail clerk	2 pkg./day	7·8
L. R. J—	53	Club operator	1 pkg./day	6·5
J. N. C—	71	Photographer	1 pkg./day ; pipe ; 5–6 cigars/day	17·9
W. F. D—	91	Telegraph inspector	3–4 cigars/day; pipe	3·0
Mean	61·1	..	..	7·14

B. *Control Specimens*

L. D—	72	Carpenter	1½ pkg./day	1·7
J. W. K—	45	Farmer	1½ pkg./day	2·1
R. L. H—	80	Painter	1 pkg./day	1·2
W. R—	61	Electrician	Pipe and cigars	1·3
F. P—	66	Cabinet maker	½ pkg./day	0·75
W. W—	40	Sawmill worker	Unknown	1·2
T. O. C—	62	Janitor	6–8 cigars/day ; 1 pkg./day	3·0
F. O. M—	71	Cook	Did not smoke	4·2
S. R. Y—	71	Groceryman	8–10 cigars/day	5·9
W. A. K—	72	Electrician	1 pkg./day	4·0
H. L. H—	63	Clerk	2 pkg./day	2·7
T. C—	55	Yard worker	¾ pkg./day	3·7
R. B—	66	Broker	1 pkg./day	5·7
A. L—	70	Laborer	½ pkg./day	11·1
G. A. R—	78	Farmer	½ pkg./day	2·1
A. H. E—	63	Nightwatchman	Pipe	9·0
J. B. C—	82	Railroadman	Unknown	1·4
H. M. N—	61	Guard	Unknown	7·7
E. E. T—	71	Insulator	1½ pkg./day	7·1
E. F—	62	Cook	2 pkg./week	2·4
W. I. G—	39	Carpenter	1 pkg./day	3·6
J. M—	49	Service Station attendant	Chewed ; dipped	4·7
J. R—	65	Railroad agent	Did not smoke	8·4
Mean	63·6	..	..	4·13

of oxygen flowed through the bomb and into an adsorption train. This step assured our obtaining most of the volatile arsenic that is generally lost in drying a tissue.

When the tissue was thoroughly dried it was removed from the bomb and the dry weight determined just as the wet weight. The specimen was next removed from the slide with arsenic-free cotton and placed back in the oxygen bomb between loose layers of cotton. The remainder of the analysis was performed exactly as described by Satterlee and Blodgett (1944).

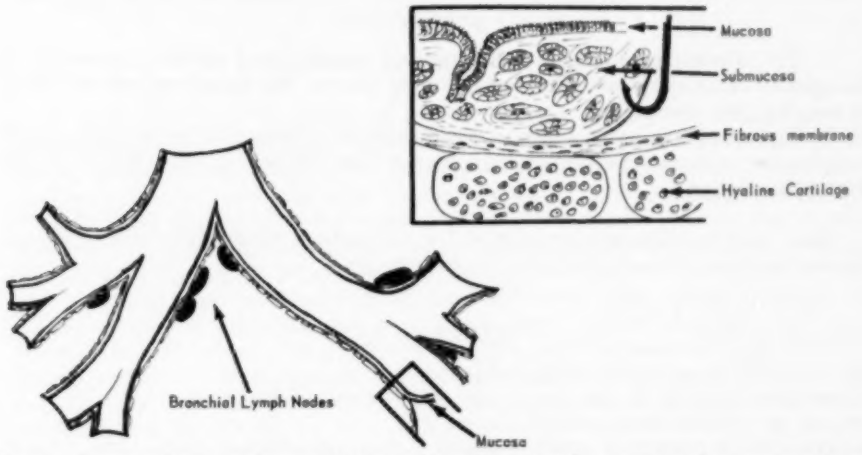


FIG. 1.—Gross specimen and insert showing the dissection plane for removing the mucosa with its underlying submucosa.

RESULTS AND DISCUSSION

Table I depicts the results of our study. The occupations and ages at the time of death were comparable in the lung cancer and control groups and there were no known industrial exposures to arsenic.

The mean value for As_2O_3 in the lung cancer tissues (7·14 μg./g. or p.p.m.) was significantly higher than that in the control group (4·13 μg./g. or p.p.m.). It appears that 5 μg. or more of As_2O_3 per gram of wet bronchial mucosa and submucosa is an excessive amount, however, it was difficult in our chemical analysis, as it was in similar studies performed in arsenical skin cancers as cited by Neubauer (1947), to determine the normal range.

Arsenic is a ubiquitous element and histories of exposures other than from tobacco smoke were not available. Bailey, Kennaway and Urquhart (1957), Satterlee (1956), and Holland *et al.* (1958) have found most American cigarette tobaccos, which are used almost exclusively in America and Eastern Europe, to contain a high arsenic content. The mean As_2O_3 value as reported by the latter investigators in 1957 was 45 μg./g. or p.p.m., which is 10–15 times the amount allowed in foods. Spot checks of several popular American cigarettes by our laboratory in 1959 showed values ranging from 18·3 to 34·2 μg./g.

Goulden, Kennaway and Urquhart (1952) and Carey, Blodgett and Satterlee (1934) have reported a high arsenic content of the dusts in urban atmospheres. The former investigators found a higher arsenic content in industrialized communities than in residential areas and they also noticed more arsenic in the dusts in the winter than in the summer months. These findings are further evidence that carbonaceous combustion products are another important source of inhaled arsenic and are another testimonial to our supposition that arsenic is the most important causal agent of lung cancer.

SUMMARY

1. The inhalation of arsenic dusts and fumes over a long period of time is now thought to be an environmental cause of lung cancer. Six recent reports are cited to confirm this observation.

2. The mean arsenic content of the bronchial mucosa and submucosa of 15 lung cancer victims was significantly higher than the mean value found in the 23 control tissues.

This investigation was supported by a Research Grant from the National Cancer Institute of the U.S. Public Health Service.

REFERENCES

Bailey, E. J.—(1957) *Brit. J. Cancer*, **11**, 54.
Idem, Kennaway, E. L. and Urquhart, M. E.—(1957) *Ibid.*, **11**, 49.
Braun, W.—(1958) *Dtsch. med. Wschr.*, **83**, 870.
Carey, F. P., Blodgett, G. and Satterlee, H. S.—(1934) *Industr. Engng Chem. (Anal.)*, **6**, 327.
Goulden, F., Kennaway, E. L. and Urquhart, M. E.—(1952) *Brit. J. Cancer*, **6**, 1.
Hess, H.—(1956) *Arch. klin Chir.*, **283**, 274.
Holland, R. H., Wilson, R. H., Acevedo, A., McCall, M. S., Clark, D. A. and Lanz, H. C.—(1958) *Cancer*, **11** : 1115.
Liebegott, G.—(1949) *Dtsch. med. Wschr.*, **74** ; 855.
Lull, L. and Wallach, A.—(1950) Montana State Department. Unpublish Data. Cited by Heuper (1956) *Publ. Hlth. Monogr.* **36**, 1.
Neubauer, O.—(1947) *Brit. J. Cancer*, **1** ; 92.
Osburn, H. S.—(1957) *Cent. Afr. J. Med.*, **3**, 215.
Roth, F.—(1957) *Germ. med. Monthly*, **11**, 172.
Satterlee, H. S.—(1956) *New Engl. J. Med.*, **254** : 1149.
Idem and Blodgett, G.—(1944) *Industr. Engng Chem. (Anal.)* **16**, 400.
Sula, J. and Zelenkova, V. *Onkologia (Czechoslovakia)* **2**, 317. Cited by Bailey, E. J. (1957) *Brit. J. Cancer* **11**, 54.

THE DOSE-RESPONSE RELATIONSHIP BETWEEN THE NUMBER OF EMBOLIC TUMOR CELLS AND THE INCIDENCE OF BLOOD-BORNE METASTASES

R. BASERGA, P. B. PUTONG, S. TYLER AND W. B. WARTMAN

From the Department of Pathology of Northwestern University Medical School, Chicago, and the Division of Biological and Medical Research, The Argonne National Laboratory, Lemont, Illinois, U.S.A.

Received for publication April 16, 1960

IT is known that the incidence of blood-borne tumour metastases may be influenced by many factors and by several experimental procedures (Baserga and Baum, 1955; Wood, 1958). One of these determining factors is the number of embolic tumor cells circulating in the blood stream. Zeidman, McCutcheon and Coman (1950) showed that the number of lung metastases in mice was roughly proportional to the number of tumor cells injected intravenously. More recently, the frequent finding of tumor cells in random samples of venous blood from tumor-bearing patients (Engell, 1955; Sandbert *et al.*, 1958; Pruitt, Hilberg and Kaiser, 1958) has indicated that a relatively large number of tumour cells may actually be present at any given time in the blood stream of these patients. These observations have prompted us to expand the investigation of Zeidman and co-workers to cover a wider range of the dose-response curve, with the objective of establishing a quantitative relationship between the number of embolic tumour cells and the incidence of metastases. Because of the quantitative conditions of the present experiment we thought it worthwhile to investigate at the same time other factors that have been said to affect the incidence of blood-borne metastases, such as the sex of the animal (Poel, 1957), the simultaneous injection of killed tumor cells (Donaldson and Mitchell, 1959) or the pre-treatment with viable tumor cells (Hackmann, 1938) as well as the response of the reticulo-endothelial system to the presence of metastases (Foulds, 1932; Druckrey *et al.*, 1939; Wartman, 1959). For these purposes, different doses of viable Ehrlich ascites tumor cells were injected into the tail vein of mice of both sexes, two groups being used to study the effects of the simultaneous injection of killed tumor cells or previous injection of viable tumor cells. The incidence and number of lung metastases in each group was determined by actual count, and the weights of the lungs, spleen, liver and kidneys were used to establish a quantitative index of the response of the reticulo-endothelial system to the presence of tumor metastases.

MATERIALS AND METHODS

$C \times AF_1$ mice, of both sexes and 4–6 months old which had been bred in the Department of Pathology of Northwestern University Medical School by Dr. Willard T. Hill, were kept in plastic cages in air-conditioned quarters, and given Rockland mouse diet and water *ad libitum*.

The tumor was Ehrlich ascites tumor, a subline of which has been propagated in this Laboratory for 5 years by weekly intraperitoneal injections to healthy

carriers. Suspensions of viable tumor cells were prepared as follows: the peritoneal fluid was aspirated 7–10 days after inoculation and centrifuged at 3000 r.p.m. for 10 minutes, the supernatant discarded and the tumor cells resuspended in sterile isotonic saline in the desired dilution. The tumour cells were counted in a hemocytometer, 5 to 10 counts being used for each dilution. Due to the difficulties involved in obtaining round numbers of tumor cells, suspensions that were as near the desired dose level as possible were used. The number of viable cells in the suspensions as determined by Schreck's method (1936), ranged between 93 and 98 per cent.

Suspensions of non-viable tumor cells were prepared as follows: 7–10-days-old Ehrlich ascites tumor was aspirated from the peritoneal cavity of healthy carriers, placed in glass tubes, centrifuged at 3000 r.p.m. for 10 minutes and the supernatant discarded. Ten per cent buffered formalin was added to the packed tumor cells in a ratio of 7 : 1 and the suspension was placed in a refrigerator at 4° C. for 12–18 hours. The formalinized cells were then centrifuged and washed 4 times with normal saline solution and finally resuspended in sterile isotonic saline in the desired dilution. Viability counts showed 100 per cent non-viable cells.

Only female mice were used to establish the dose-response curve. The number of tumor cells injected and the number of animals in each group are shown in Table I. For the second part of the experiment, on the incidence of metastases

TABLE I.—*Incidence of Metastases in CAF_1 Female Mice Injected Intravenously with Ehrlich Ascites Tumor Cells*

Number of tumor cells injected ± *S*	*N*		% of mice with metastases		Number of metastases	Number of metastases per mouse	
None	25		0·0		0	0·000	
905 ± 170	19	27	10·5	7·4	2	0·105	0·074
14,390 ± 700	8		0·0		0	0·000	
93,200 ± 6,800	16		25·0		4	0·250	
382,000 ± 80,000	18		33·3		8	0·444	
597,000 ± 90,000	44		52·3		37	0·864	
747,000 ± 110,000	26		80·8		58	2·192	
928,000 ± 76,000	16		100·0		97	6·062	
1,180,000 ± 88,000	8		100·0		114	14·250	
1,654,000 ± 140,000	20		100·0		815	40·750	
1,885,000 ± 350,000	10		100·0		—	>200	
4,526,000 ± 180,000	14		100·0		—	>200	
6,750,000 ± 120,000	11		100·0		—	>200	
8,696,000 ± 430,000	6		100·0		—	>200	

S = standard deviation.
N = number of mice.

in mice previously treated with viable or non-viable tumor cells, only male mice were used. The number of tumor cells injected and the number of animals in each group are shown in Table III. All injections, either of viable or non-viable cells, were made into the tail vein, using a 27-gauge needle and a calibrated syringe. About half the injections resulted in local growths at the injection site in the tail or at the root of the tail. All animals that showed the slightest evidence of local growths were discarded, and were not included in the computations.

Except when otherwise stated, the mice were sacrificed by cervical dislocation 30 days after the injection of tumor cells. The body weight and the weights of

the lungs, liver, spleen and left kidney were determined for each animal. The lungs were examined and the number of grossly visible metastases counted by two different observers. Precise counts were not possible when the number of metastases in both lungs was above 200.

The number of tumor cells in a given weight of packed Ehrlich ascites tumor was determined as follows: 5 ml. of tumor cell suspension, from 8-day-old peritoneal growths were measured in a calibrated pipette and the number of cells per ml. was determined as usual with a hemocytometer. After centrifuging and discarding the supernatant, the packed tumor cells were weighed on an analytical balance, the weight obtained being taken as the weight of the number of cells contained in 5 ml. of tumor suspension. The procedure was repeated on 5 different animals, and the results were averaged.

RESULTS

1. *Dose-response relationships*

Table I shows the incidence of lung metastases in CAF_1 female mice following intravenous injection of Ehrlich ascites tumor cells. Animals alive on the 30th day of the experiment were sacrificed. Other animals were autopsied on the day of death. All animals of the groups receiving less than 1,180,000 cells were alive on the 30th day, and the last 5 groups had mean survival times equal to 26, 20, 16, 15 and 14 days respectively. The incidence of metastases below 100 per cent when plotted on probability paper, was linearly related to dose (Fig. 1). This indicates that the distribution of susceptibilities to Ehrlich ascites tumor cells is approximately normal with least square estimates of mean and standard deviation equal to 512,000 and 394,000 cells respectively. The relationship between average number of metastases per mouse and dose, for groups in which a tumor count could be made, is shown in Fig. 2 and is definitely nonlinear. However, the difference between the trend seen at small doses and that characterizing large dose groups suggests that at least two processes may influence the pattern seen in Fig. 2. It is of interest to note that for doses equal to or less than 600,000 cells, the relation between variables is essentially linear; while for doses exceeding 600,000 cells the pattern of points accelerates even faster than a simple exponential function. Also for doses not exceeding 600,000 cells the group incidence predicted, based on Poisson expectations with the observed number of metastases per mouse as mean value, agrees closely with the observed incidence within groups. On the contrary, for doses above 600,000 cells, the expectations greatly deviate from the observed.

Since the lung is the principal site of establishment and growth of Ehrlich ascites tumor cells injected intravenously, a weight change in this organ should reflect the severity of the insult sustained by the organism as the dose is increased and provide a means of interpolation between the experimentally controlled dosages. The increase in lung weight with injected dose (Table II) was found to be a moderately sensitive indicator. Since the average weight of the lungs for groups injected with 382,000 cells or less seemed to vary about the mean of the controls in a random fashion, the mean weight of the lungs of the controls was taken as the weighted average of these groups and was found to be 173·2 mg. A linear relationship between mean lung weight as per cent of the control value and dose exists over a range of doses extending from 600,000 cells to approximately

2 million cells (Fig. 3). Lung weights for injected doses above this range tend toward an asymptotic or probably an anatomical limiting value which is in the neighborhood of 700 mg. Within this range of doses, the magnitude of the slope of the least square line indicates that the lung weight increases at a constant rate of 0·165 grams per 1000 cells injected. The equation of the least square line is :

$$L = 3{\cdot}55 + 0{\cdot}165D, \qquad 6 \times 10^2 D\ 2 \times 10^3 \tag{1}$$

Where L is the mean lung weight in per cent of the control value and D is the dose in thousands of injected cells.

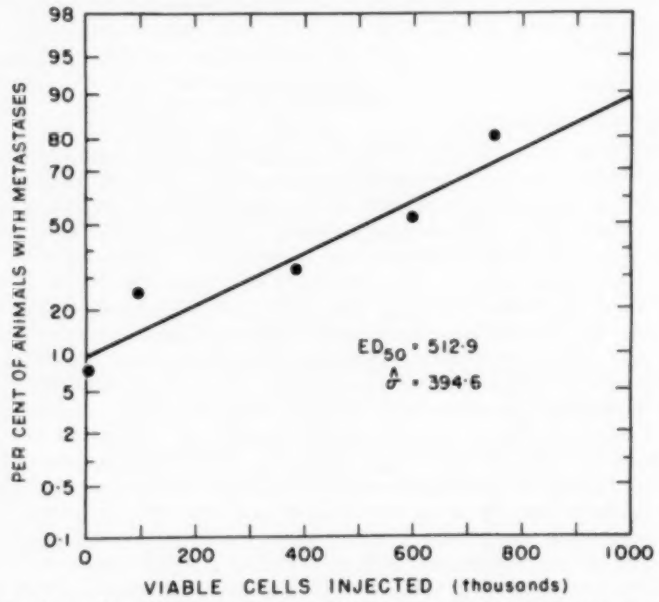


FIG. 1.—Probit transformation of the dose-incidence curve. Per cent of animals with lung metastases, CAF_1 female mice injected intravenously with Ehrlich ascites tumor cells. Slope = 0·002534 ± 0·000498 ; $ED_{50} = 512{\cdot}9 \pm 51{\cdot}2 \times 10^3$ cells.

Although the dependence between lung weight and mean number of metastases per animal does not allow a simple explanation, an empirically derived functional relationship between these variables is presented in Fig. 4. A log-log plot of the variables shows a linear relationship between their log transforms. This relationship at doses exceeding 382,000 cells is expressed by the power law

$$M = 3{\cdot}689(10^{-10})\, L^{4{\cdot}622} \tag{2}$$

where M is the mean number of metastases per animal and L is the mean lung weight in per cent of the control weight. From equations (1) and (2), an empirical expression of the dependence between number of metastases and dose can readily be determined.

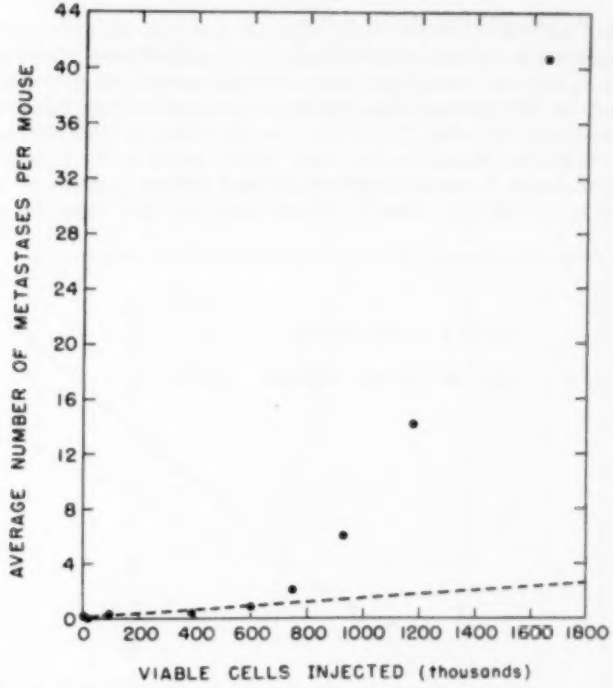


FIG. 2.—Relationship between average number of lung metastases per mouse and number of tumor cells injected. CAF_1 female mice injected intravenously with Ehrlich ascites tumor cells.

TABLE II.—*Mean Weights of Lungs, Spleen, Liver and Left Kidney of CAF_1 Female Mice Injected Intravenously with Ehrlich Ascites Tumor Cells*

Number of tumor cells injected	Body weight			Lungs weight			Liver weight			Spleen weight			Kidney weight		
	n	$\bar{X}$	$S\bar{x}$	n	$\bar{X}$	$S\bar{x}$	n	$\bar{X}$	$S\bar{x}$	n	$\bar{X}$	$S\bar{x}$	n	$\bar{X}$	$S\bar{x}$
None	25	26·8	0·3	25	182	2	14	1529	61	25	127	4	25	174	2
905	19	25·9	0·5	19	181	4	19	1301	29	19	132	3	19	165	4
14,390	8	25·8	1·2	8	161	4	8	1334	33	8	124	7	8	152	4
93,200	16	27·9	0·4	16	178	5	8	1472	43	16	159	7	16	173	3
382,000	18	26·0	0·3	18	154	4	18	1274	38	18	125	12	18	159	3
597,000	44	26·5	0·3	44	182	4	16	1539	80	42	158	6	23	181	4
747,000	25	27·1	0·6	26	225	11	9	1485	65	23	229	27	10	191	5
928,000	16	26·2	0·5	16	277	11	—	—	—	16	224	12	10	185	3
1,180,000	8	24·6	1·6	8	344	41	—	—	—	7	241	23	—	—	—
1,654,000	20	25·6	0·4	10	451	17	17	1656	41	19	327	12	19	183	4
1,885,000	10	25·6	1·0	10	567	36	—	—	—	6	338	40	—	—	—
4,526,000	14	24·7	0·8	14	572	34	6	1895	46	6	361	14	6	175	7
6,750,000	11	25·1	0·8	11	665	36	—	—	—	—	—	—	—	—	—
8,696,000	6	25·6	0·9	6	687	48	—	—	—	3	327	12	—	—	—
Subq. injection	14	26·9	0·5	14	205	7	2	1925	145	22	504	23	4	189	12

n = number of measures included in mean.
$\bar{X}$ = mean, in g. for body weight, in mg. for lungs, spleen, liver and kidney.
$S\bar{x}$ = standard error of the mean.
Subq. injection : a group of mice with huge tumor growths at the root of the tail.

2. *Response of the reticulo-endothelial system*

Even though not a single metastasis was found, either grossly or histologically, in any organ except the lung, a significant response to the presence of tumor cells was noted in the spleen. The weight of the spleen highly correlates with number of tumor cells injected (Table II). In the liver the correlation is partially obscured by variations related to the body weight while in the kidney, a statistically significant trend between weight and dose is not present in the sample. Spleen weight and dose are linearly related (Fig. 3), and thus, by equation 1,

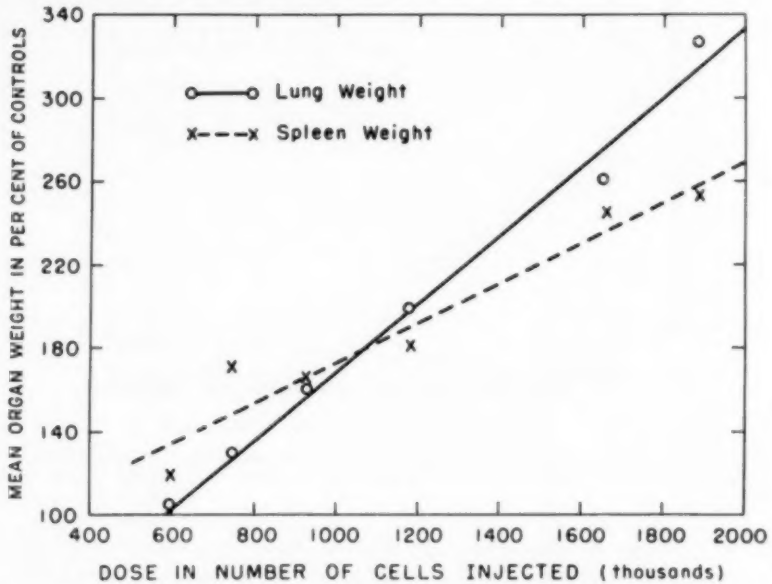


FIG. 3.—Lung and spleen weights versus dose in CAF_1 female mice injected intravenously with Ehrlich ascites tumor cells. Weight of control lungs: 173·2 mg.; slope = 0·1651 ± 0·0094, intercept 3·55 ± 11·88. Weight of control spleen: 133·6 mg.; slope = 0·0962 ± 0·0137, intercept = 77·28 ± 17·17.

a straight line relationship between the weights of the spleen and lungs is implied. In those mice in which an improper intravenous injection resulted in a huge growth at the root of the tail (Table II), the weight of the spleen was, on the average, 3·7 times the weight of controls. This indicates that the spleen may respond to the presence of tumor cells regardless of the site of tumor growth.

3. *The results in male mice*

These are shown in Table III, from which it is apparent that the incidence of metastases in male mice is considerably lower than in female mice, the 5 per cent critical level being used as a measure of significance. It will also be noted that when killed tumor cells are injected simultaneously with viable cells, the incidence of lung metastases in male mice increases.

When male mice, are injected, 80 days apart, with two similar doses of viable tumor cells, the incidence of lung metastases is about twice that observed in mice injected with a single dose. This seems to indicate that previous treatment with viable tumor cells does not alter the response of the host to a second injection of viable tumor cells.

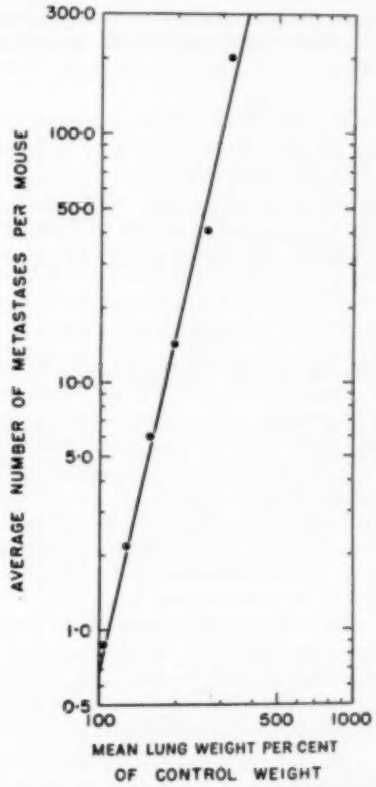


FIG. 4.—Log-log plot of lung weight versus mean number of metastases in CAF_1 female mice injected intravenously with Ehrlich ascites tumor cells. Slope = 4·622 ± 0·208, intercept = − 9·433 ± 0·471.

4. *The estimated number of tumor cells surviving at the site of arrest*

With the procedure outlined in Methods and Materials, it was found that 1 ml. of EAT contained on the average, $139{,}84 \times 10^6$ cells, and that after discarding the supernatant, this number of packed cells weighed 278 mg. (mean of 5 trials). This meant that 1 mg. of packed EAT cells contained an average of 503,000 cells. Determinations of the percentage dry weight of the lungs showed that there was little difference between the control lungs (19 per cent dry weight) and the heaviest lungs filled with tumor (18 per cent dry weight). As the mean weight of the lungs

of CAF_1 female mice was found to be 173·2 mg., any increase in lung weight above this figure will give a rough approximation of the number of tumor cells present in the lungs. Parallel studies using tritiated thymidine (Baserga, Kisieleski and Halvorsen, 1960) have shown that EAT cells injected intravenously in CAF_1 female mice grow without a latent period, at a doubling time of 20 hours, and with 100 per cent of the cells dividing every 20 hours. It is then possible to calculate from these data the approximate number of tumor cells injected that actually survived at the site of arrest and developed into a tumor metastasis. This may be expressed by the equation

$$N_0 = \frac{N_t}{2^x} \tag{3}$$

where N_0 is the number of surviving tumor cells at the time of injection, N_t is the number of tumor cells calculated from the lung weight, and x is the number of doubling times between injection and death. Calculations have been performed for the last 6 groups and the results are shown in Table IV. These indicate that the number of injected tumor cells surviving at the site of arrest is less than one in one thousand.

DISCUSSION

The advantages of using ascites cell suspensions in the study of blood-borne metastases have been pointed out in 1936 by Warren and Gates, and, more recently by Ambrus *et al.* (1956). The advantages are mainly three, i.e. most of the tumor cells are viable, little or no stroma is injected with the tumor cells, and it is possible to reduce to a minimum the contamination with cellular debris which is unavoidable with minced tumor tissue. These advantages are particularly important in quantitative studies as shown in the present experiment (Table III), in which the simultaneous injection of killed tumor cells increased the incidence of lung metastases produced by the intravenous injection of viable tumor cells.

Although the technique of intravenous injection of tumor cells still remains an artificial procedure when compared to the observation of spontaneous blood-borne metastases (Baserga and Shubik, 1955), it should be noted that according to Wallace (1956), metastases from intravenously injected cells can be obtained only with those tumors that are also capable of spontaneous metastases. With these qualifications, the following considerations may be made.

1. *The dose-response relationship*

When suspensions of EAT cells are injected intravenously in CAF_1 mice, in doses ranging from as few as 905 cells to as many as $8{\cdot}7 \times 10^6$ cells, the incidence of lung metastases increases as previously pointed out by Zeidman *et al.* (1950), with increasing doses. The relationship between dose and mean number of metastases, however, is linear only up to a dose of 600,000 cells, but for doses exceeding 600,000 cells the relationship deviates from linearity and accelerates even faster than a simple exponential function. As the tumor cell suspensions used in the present experiments were all prepared, by dilution, from an original pool, it must be assumed that the tumor cell population had a constant per cent composition in the various doses. Then, the changing slope of the dose-response curve indicates that the establishment of a metastatic growth does not depend solely on the presence of favored cells capable of survival at the site of arrest,

but that, at least with doses exceeding 600,000 cells, other factors besides the composition of the tumor cell population must be operating. A possible explanation may be found in the experiments of Kaziwara (1954), who, by employing small doses of cells in intraperitoneal inocula, was able to transform a hyperdiploid Ehrlich tumor line into a near-tetraploid strain. Small doses then may have the effect of selecting only a few favored cells, whereas, with larger doses, different clones of cells may survive at the site of arrest. In such a case, our results would not necessarily be at variance with the findings of Rabotti (1959), who claimed that metastases differ from primary growths by having a higher number of polyploid cells.

It is interesting to compare our results with those obtained by Warner and James (1959), who studied the dose-response relationship of EAT cells injected intraperitoneally. They found too that the dose-response curve departed from exponentiality, and that the results could be best summarized by plotting the distribution of sensitivities of mice against the dose, as we have done in Fig. 1. By comparing the two distributions of sensitivities, it would appear that whereas 850 cells are required when injected intraperitoneally to produce tumor growths in 50 per cent of the animals, a mean number of 512,000 cells must be injected intravenously to obtain the same percentage incidence of lung metastases. As doses increase, however, the differences seem to disappear, and approximately 1,000,000 cells are required to produce a 100 per cent incidence of either lung metastases or peritoneal growths.

From the present data, it may also be stated that, at doses exceeding 382,000 cells, a linear relationship exists between the log transforms of lung weight in percent of control weight and mean number of metastases, so that the increase in lung weight may be used as an indicator of the mean number of metastases. This is further confirmed by the linear relationship existing between number of tumor cells injected and per cent increase of lung weight.

2. *The response of the reticulo-endothelial system*

Several authors, in the past, have suggested that the reticulo-endothelial system participates in the process of metastatic growth. Foulds (1932) found that the incidence of metastases in the lungs, liver and spleen from Brown-Pearce tumors increased considerably when the rabbits had been previously injected with trypan blue. Brouwer (1938) obtained similar results with a single injection of 1 c.c. of Thorotrast, but, when using higher doses of Thorotrast, he could not show any increase in susceptibility. The increase in the incidence of metastases brought about by whole-body irradiation of the animal host (Cirio and Balestra, 1930; Flaks and Grynkraut, 1934) has also been attributed to the depressing action of irradiation upon the reticulo-endothelial system, and similar views were expressed to explain the favorable action of cortisone on metastases (Pomeroy, 1954). Conversely, stimulation of the reticulo-endothelial system by subcutaneous injections of carotin was held to be responsible for the inhibition of growth of two different transplantable rat tumors, the Flexner-Jobling carcinoma and the Jensen sarcoma (Stern and Willheim, 1935). The present data definitely indicate a response of the reticulo-endothelial system, in the form of hyperplasia to the presence of tumor cells in the animal host. In fact, the linear correlation between the spleen weights and the lung weights even suggests that the weight

TABLE III.—*Incidence of Lung Metastases in CAF_1 Mice Injected Intravenously with Ehrlich Ascites Tumor Cells*

	Number of viable cells injected (thousands)	Number of killed cells injected (thousands)	2nd injection. Number of viable cells (thousands)	N	Number of animals with metastases	% with tumors	σ	* Expected % with tumors	Weighted mean of tumors per animal
Females (weighted average of 597·0 and 747·0 groups)	652·7	0	0	70	44	62·9	5·8	74·3	1·36
Males	661·0	744·0	0	47	16	34·0	6·9	37·5	0·47
Males	661·0	0	0	36	5	13·9	5·8	13·1	0·14
Males	661·0	0	684·0	39	11	28·2	7·2	24·4	0·28

N = number of animals.
σ = standard deviation of the per cent.
* = expected number determined using the Poisson distribution.

TABLE IV.—*The Number of Tumor Cells Surviving at the Site of Arrest*

Number of tumor cells injected (thousands)	Exit (in days)	Number of doubling times	Mean lung weight (mg.)	Difference from control weight	Number of cells in difference	Surviving cells	Proportion cells surviving
1885	20	24·0	567·00	394·0	198×10^6	12	$0·06 \times 10^{-4}$
4526	16	19·2	571·79	398·79	201×10^6	334	$0·74 \times 10^{-4}$
6750	15	18·0	664·55	491·55	247×10^6	942	$1·40 \times 10^{-4}$
8696	14	16·8	686·67	513·67	259×10^6	2270	$2·61 \times 10^{-4}$

Mean control weight was taken as 173 mg.
Doubling time of EAT = 20 hours.
1 mg. of packed EAT = 503,000 cells.

$$\text{Number of surviving cells} = \frac{\text{Number of cells in difference}}{2^{(\text{generations})}}$$

of the spleen may be used as an indicator of the amount of tumor present in the host. Whether the hyperplasia of the reticulo-endothelial system can be regarded as favorable to the host or not, our data do not indicate.

3. *The results obtained in male mice*

We have already mentioned that the simultaneous injection of killed tumor cells increases the incidence of metastases and that the pre-treatment of mice with viable tumor cells does not have any influence on the number of metastases induced by a subsequent injection of viable tumor cells. Perhaps of more interest is the striking difference in the incidence of metastases, after intravenous injection of EAT cells, between male and female CAF_1 mice. A high incidence of metastases from mammary carcinomas has been reported in estrogen-treated rats (Nelson, 1944). Poel (1957) has found that the incidence of lymph node metastases from chemically induced skin tumors was higher in female than in male mice. To bring out these differences, it is probably necessary to use relatively small doses, as previously suggested by Gross (1942), who had found sex differences in the response to the subcutaneous or intraperitoneal inoculation of a transplantable sarcoma in mice, only when using small doses. These results should not be construed, however, as implicating a higher susceptibility of females to metastases in general, as the reason for the difference may well reside in the particular tumor. The results show, however, the advantages of intravenous injections of relatively small doses of cells in the investigation of the various factors that influence the incidence of blood-borne metastases (Wood, 1958).

4. *The number of tumor cells surviving at the site of arrest*

It has been known for a long time that the majority of tumor cell emboli fail to survive at the site of arrest (Goldmann, 1897 ; Iwasaki, 1915 ; Zeidman *et al.*, 1950), and recent experiments in this Laboratory using tritiated thymidine to label injected tumor cells showed that the percentage of Ehrlich ascites tumor cells that survive at the site of arrest is not above 8 per thousand (Baserga *et al.*, 1960). Calculations based on the present experiments (Table IV) indicate that the 8 per thousand figure should be revised downward, and that, in all probability, at least with EAT, each metastasis originates from a single tumor cell.

The data used in these calculations are not all of the same accuracy. The doubling time of EAT, 20 hours, and therefore the number of doubling times in each group, are known with considerable precision, and the number of tumor cell per mg. of packed tumor cells can be considered reasonably accurate, the standard deviation of the count not exceeding 10 per cent. The least precise of the data is the actual number of tumor cells present in the lungs, which is based on the difference from the control weight, that is, on the assumption that the amount of normal lung tissue remains constant. Actually, normal lung tissue is in part replaced by tumor tissue especially when the number of metastases is high. As we have used the lung weight of normal animals as the base line, the actual amount of tumor present in the lungs, either expressed in weight or in number of tumor cells, must then be revised upward from the figures given in Table IV. The difference, however, cannot be more than 25 per cent, and the resulting corrections in the number of tumor cells surviving at the site of arrest would not change the two conclusions that can be drawn from these calculations,

i.e., that the number of surviving cells is in the order of one or less per thousand and that most of the metastatic growths originate from single cells.

SUMMARY

The dose-response relationship between the number of intravenously injected tumor cells and the number of lung metastases was investigated in CAF_1 mice using suspensions of viable Ehrlich ascites tumor cells. The correlation was linear for doses up to 600,000 cells, but with higher doses the relation between variables increased even faster than a simple exponential function, thus suggesting a two-fold mechanism in the establishment of tumor metastases. The increase in lung weight, for doses exceeding 382,000 cells, was linearly correlated to the number of injected cells, and its log transforms were linearly correlated to the logarithms of the mean number of metastases. At equal dose levels, the incidence of metastases was much higher in female than in male mice, and the incidence in males was also increased by the simultaneous injection of killed tumor cells. Previous treatment with viable tumor cells did not alter the response of the host to the subsequent injection of a second dose of viable tumor cells. The weight of the spleen was linearly correlated to the weight of the lungs, thus suggesting a quantitative response of the reticulo-endothelial system to the presence of tumor in the lung.

We wish to acknowledge our debt to Dr. Willard T. Hill for his generous help, and to Miss Annette Serpico, for secretarial help.

This work was performed in part with the aid of a grant (P-142) from the Illinois Branch of the American Cancer Society, and in part under the auspices of the U.S. Atomic Energy Commission.

REFERENCES

Ambrus, J. L., Ambrus, C. M., Byron, J. W., Goldberg, M. E. and Harrisson, J. W. E.—(1956) *Ann. N.Y. Acad. Sci.*, **63**, 938.
Baserga, R. and Baum, J.—(1955) *Cancer Res.*, **15**, 52.
Idem, Kisieleski, W. and Halvorsen, K.—(1960) *Ibid.*, in press.
Idem and Shubik, P.—(1955) *Science*, **121**, 100.
Brouwer, P.—(1938) *Beitr. klin. Chir.*, **168**, 616.
Cirio, L. and Balestra, G.—(1930) *Pathologica*, **22**, 451.
Donaldson, D. M. and Mitchell, J. R.—(1959) *Proc. Soc. exp. Biol. N.Y.*, **101**, 204.
Druckrey, H., Hamperl, H., Herken, H. and Rarei, B.—(1939) *Z. Krebsforsch.*, **48**, 451.
Engell, H. C.—(1955) *Acta chir. scand.*, Suppl. 201, 1.
Flaks, J. and Grynkraut, B.—(1934) *Acta cancrol., Bp.*, **1**, 76.
Foulds, L.—(1932) *Sci. Rep. Cancer Res. Fd. Lond.*, **10**, 21.
Goldmann, E. E.—(1897) *Beitr. klin. Chir.*, **18**, 595.
Gross, L.—(1942) *Proc. Soc. exp. Biol. N.Y.*, **49**, 67.
Hackmann, C.—(1938) *Z. Krebsforsch.*, **48**, 169.
Iwasaki, T.—(1915) *J. Path. Bact.*, **20**, 85.
Kaziwara, K.—(1954) *Cancer Res.*, **14**, 795.
Nelson, W. O.—(1944) *Yale J. Biol. Med.*, **17**, 217.
Poel, W. E.—(1957) *J. nat. Cancer Inst.*, **19**, 1013.

POMEROY, T. C.—(1954) *Cancer Res.*, **14**, 201.
PRUITT, J. C., HILBERG, A. W. AND KAISER, R. F.—(1958) *New Engl. J. Med.*, **259**, 1161.
RABOTTI, G.—(1959) *Nature, Lond.* **183**, 1276.
SANDBERG, A. A., MOORE, G. E., CROSSWHITE, L. H. AND SCHUBARG, J. R.—(1958) *Cancer*, **11**, 1180.
SCHREK, R. A.—(1936) *Amer. J. Cancer*, **28**, 389.
STERN, K. AND WILLHEIM, R.—(1935) *Z. ges. exp. Med.*, **97**, 354.
WALLACE, A. C.—(1956) *Brit. J. Cancer*, **10**, 724.
WARNER, P. and JAMES, A. T.—(1959) *Ibid.*, **13**, **288**.
WARREN, S. AND GATES, O.—(1936) *Amer. J. Cancer*, **27**, 485.
WARTMAN, W. B.—(1959) *Brit. J. Cancer*, **13**, 389.
WOOD, S., Jr.—(1958) *Arch. Path.*, **66**, 550.
ZEIDMAN, I., MCCUTCHEON, M. AND COMAN, D. R.—(1950) *Cancer Res.*, **10**, 357.

FURTHER STUDIES RELATING TO THE IMPLICATIONS OF RADIATION SURVIVAL CURVE DATA FOR TREATMENT OF CBA MOUSE LEUKAEMIA BY WHOLE-BODY IRRADIATION

H. B. HEWITT AND C. W. WILSON

From the Westminster School of Medicine, London, S.W.1

Received for publication February 9, 1960

THE CBA mouse leukaemia used in the present experiments was described previously (Hewitt, 1958). Evidence was then presented to show that the CBA host mice to which the leukaemia was transplanted exhibited no detectable immunological reactivity to the leukaemia cells. This host-tumour system therefore provides an ideal model for the examination of certain radiobiological concepts pertinent to the radiotherapy of autologous tumours. The annals of clinical radiotherapy are in themselves sufficient evidence that no immunological resistance against autologous tumours in the human host can be relied upon to destroy viable malignant cells that have withstood the best endeavours of the radiotherapist, and it cannot be too strongly emphasised that animal host-tumour systems used to provide data relevant to clinical radiotherapy should be free from complicating immunological factors. Scott (1958) and Klein (1959) have reviewed some of the complexities and fallacies that have often confused the interpretation of radiobiological data obtained from experiments in which immunologically reactive hosts were used to detect viable malignant cells that have survived irradiation.

Using the CBA leukaemia host-tumour system referred to, Hewitt and Wilson (1959*a*) determined a radiation survival curve for the leukaemia cells irradiated *in vivo*. A linear relationship between dose of radiation and log survival rate was demonstrated up to the maximum dose of radiation used—2000 r of ^{60}Co gamma rays (corresponding to a survival rate of about $1/10^5$). The slope of the curve indicated a mean lethal dose of radiation (D_0) of 165 r. Later evidence (Hewitt and Wilson, 1959*b*) showed that the leukaemia cells infiltrating the livers of advanced leukaemic mice breathing air had a radiosensitivity compatible with their having been in a moderately well-oxygenated environment at the time of irradiation; when irradiated under anoxic conditions, the cells were shown to be more radioresistant by a factor of approximately 2·3.

It will be appreciated that the survival curve data can be used to calculate the minimum dose of radiation theoretically required to "cure" mice bearing leukaemia cell populations of known size, provided that these cells have the same radiosensitivity as the cells irradiated in the survival curve experiments. The present experiments were designed to test directly our ability to predict "cure" rates in this way, and to explore such factors as might be expected to disqualify the simple application of the survival curve envisaged.

MATERIALS AND METHODS

Mice.—CBA mice of either sex bred in this laboratory by sib-mating were used exclusively ; the mice were 2–4 months old when used for experiment.

Leukaemia.—This was a lymphocytic type of leukaemia which arose spontaneously in a male mouse of the CBA colony which provided all the mice used in the experiments. The leukaemia was in its 74th to 108th serial passage when used in the present experiments. A detailed account of the characteristics of the leukaemia, and of a transplantation bio-assay method for determining the mean number of morphologically intact leukaemia cells required to convey leukaemia to half a group of injected mice (the TD50), was given previously (Hewitt, 1958). An average TD50 of 2 cells was obtained in a series of 6 such assays of cells from untreated leukaemic mice.

Irradiation.—For the whole-body irradiation of groups of mice, these were exposed in a " Perspex " box to ^{60}Co gamma radiation delivered via a single field in a single dose uniform to $\pm$ 5 per cent over a period of 16 hours. The irradiated mice received 1·0–1·5 $\times$ 10^6 nucleated isologous bone marrow cells intravenously within 2 hours of the end of irradiation.

" Radiation-killed cells " consisted of freshly-prepared single-cell suspensions of leukaemia cells in 5 per cent CBA mouse serum in Tyrode solution (density, 8 $\times$ 10^6 cells/ml.) which had been exposed immediately before use to a total uniform dose of 6000 r ^{60}Co gamma radiation delivered over a period of 24 minutes. The absence of viable leukaemia cells from the suspension was proved by the injection of aliquots into highly susceptible mice, all of which failed to develop leukaemia.

EXPERIMENTS AND RESULTS

The effect of radiation-killed cells on the growth of viable leukaemia cells

To measure survival ratios among cells irradiated *in vivo*, as was done for determination of the survival curve already referred to (Hewitt and Wilson, 1959*a*), single-cell suspensions of leukaemia cells were prepared from the infiltrated livers of leukaemic mice within 30 minutes of their exposure to whole-body irradiation, and these were injected intraperitoneally into groups of normal mice. It is clear that adequate interpretation of the survival curve determined from such data requires information concerning the possible influence of the radiation-killed cells on the ability of the residual viable cells to convey leukaemia. Such an influence has been described previously for other tumour-host systems (Révész, 1958).

In the present case, an inhibitory influence of the radiation-killed cells would lead to overestimation of the proportion of leukaemia cells deprived of their reproductive integrity by the radiation. The existence of such an influence was sought in the following experiment : a counted single-cell suspension of leukaemia cells from an untreated leukaemic mouse was assayed by the intraperitoneal injection of selected dilutions of the suspension into groups of 5 or 6 normal mice ; the assay was performed identically in two parallel series of mice ; immediately after injection of the viable cells, each mouse of one series was given a further intraperitoneal injection, of 0·1 ml. of serum-Tyrode medium ; each mouse of the other series received 0·1 ml. of a suspension in the same medium of

800,000 radiation-killed leukaemia cells ; the injected mice were observed for two months (a period twice as long as the longest latent period observed for the development of leukaemia following the injection of minimal numbers of viable cells) and the incidence of leukaemia in each injected group of each series was recorded. The TD50 value for each series was calculated from the mortality data by the method of Reed and Muench (1938). The results of the experiment are recorded in Table I, from which it will be seen that the TD50 values for the

TABLE I.—*Assay of Viable Leukaemia Cells* (a) *Injected Alone ;* (b) *Injected with Preponderence of Radiation-killed Cells*

Mean dose of viable cells	Ratio of viable to killed cells (*b*)	Incidence of leukaemia (*a*)	Incidence of leukaemia (*b*)
125	$1 : 64 \times 10^2$	6/6	5/6
12·5	$1 : 64 \times 10^3$	5/6	5/6
1·25	$1 : 64 \times 10^4$	0/5	3/5
0·125	$1 : 64 \times 10^5$	0/6	0/6
	TD50 :	5 cells	1·9 cells

two series were not significantly different from one another or from the average figure (2·0) obtained for a previous series of assays in normal mice. It may be added that no significant difference of survival times of the mice was observed for corresponding groups of the two series.

It is concluded from this experiment that a preponderance of radiation-killed cells, intimately mixed with the viable cells, does not influence the capacity of the latter to reproduce the full disease in mice to which they are transplanted. Six mice which received $1{\cdot}6 \times 10^6$ radiation-killed cells failed to develop leukaemia after prolonged observation.

Assay of leukaemia cells in whole-body irradiated mice

When leukaemia cells are irradiated *in vivo* by whole-body irradiation of leukaemic mice, and the treated mice are retained, it is conceivable that, in addition to those cells which lose their reproductive integrity by a direct action of radiation, others may do so later, as the result of indirect cytotoxic influences resulting from relatively persistent constitutional changes induced in the host by the whole-body irradiation. If such an indirect influence is active, the survival ratios determined among the cells removed from the mouse very soon after irradiation would fail to indicate the total damage to the malignant cell population which would ensue among cells allowed to remain in the irradiated host. In these circumstances, survival curves determined as previously described (Hewitt and Wilson, 1959*a*), in which a sample of the irradiated cell population was removed from the leukaemic mouse almost immediately after irradiation, would provide an unduly pessimistic estimate of the dosage requirements for successful radiotherapy. The following experiment was designed to reveal any inimical influence, capable of destroying viable leukaemia cells, which might be active in whole-body irradiated mice.

A suspension of viable leukaemia cells was assayed in a control series of normal mice and, in parallel, in an equivalent series of mice which had received 2000 r whole-body ^{60}Co gamma radiation followed by intravenous isologous bone marrow. The assay was performed less than 6 hours after exposure of the irradiated mice

TABLE II.—*Assay of Leukaemia Cells in Normal Mice and in Whole-body Irradiated Mice*

Mean number viable cells injected	Incidence of leukaemia	
	In normal mice	In irradiated mice
1000	5/5	4/4
100	5/5	5/5
10	2/4	3/5
1	1/4	1/5
TD50 :	5·6 cells	4·6 cells

had been completed. The results of the two assays, given in Table II, showed that there is no significant difference between the TD50 values obtained in the normal and irradiated mice. Also there was no significant difference in the survival times of mice in corresponding groups of the two series. It is clear that no very persistent constitutional changes are present in the irradiated mice which interfere with the reproductive capacity of cells which have survived the direct lethal effects of radiation. These findings support the hypothesis that the survival curve data can be used to predict dosage requirements for the successful radiotherapy of mice harbouring malignant cell populations of known size.

Dose of whole-body radiation required to "cure" mice bearing known numbers of leukaemia cells

Groups of 7 CBA male mice were injected intraperitoneally with dilutions of a single-cell suspension of leukaemia cells prepared from an untreated leukaemic mouse. The mean dose of morphologically intact leukaemia cells per mouse for each group is shown in the first column of Table III. Within one hour of injec-

TABLE III.—*Observed and Expected Incidences of Leukaemia in Groups of Mice Bearing Known Mean Numbers of Leukaemia Cells at Time of Exposure to 1600 r ^{60}Co Gamma Radiation*

Mean number of leukaemia cells injected	Mean number of cells expected to survive in mice after irradiation	Expected leukaemia incidence (per cent)	Observed leukaemia incidence
$1·1 \times 10^5$	110	100	6/6 (100%)
$1·1 \times 10^5$	11	86	6/7 (86%)
$1·1 \times 10^4$	1·1	34	1/7 (14%)
$1·1 \times 10^3$	0·11	10	0/7 (0%)
$1·1 \times 10^2$	0·011	0	0/7 (0%)

tion, all the potentially leukaemic mice were exposed to 1600 r whole-body ^{60}Co gamma radiation delivered in a single uniform dose over a period of 16 hours. Within a few hours of the end of irradiation each mouse received intravenously approximately 10^6 isologous nucleated marrow cells. The mice were then retained for observation for a period of at least 4 months, during which time all deaths among the mice were investigated by post-mortem examination.

One mouse injected with the highest cell dose was eliminated from the results because it died early of the effects of radiation. All the other deaths were due to widespread infiltration of viscera with leukaemia cells, indicating that the dose

of whole-body radiation had failed to eliminate the total population of leukaemia cells present in such mice at the start of irradiation. The incidence of leukaemia deaths in each group is recorded in the last column of Table III. The details given in the 2nd and 3rd columns of Table III were derived from previous data as follows. From the survival curve for the leukaemia cells irradiated *in vivo* (Hewitt and Wilson, 1959*a*), which is reproduced in Fig. 1, it is seen that a dose of 1600 r ^{60}Co gamma radiation gave a survival ratio of 10^{-4}; that is, only 1/10,000 of

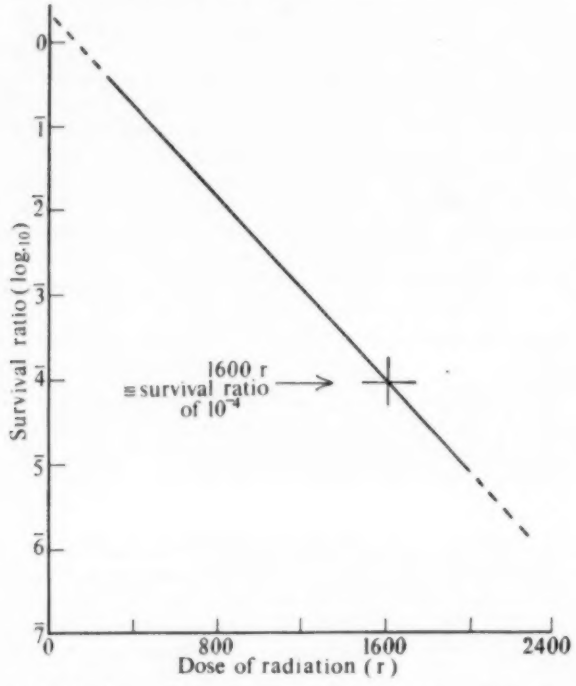


FIG. 1.—Survival curve for CBA mouse leukaemia cells irradiated *in vivo*.

the irradiated leukaemia cells retained their reproductive integrity after exposure to that dose of radiation. The figures in the 2nd column of Table III are the mean numbers of leukaemia cells per mouse expected to retain their reproductive integrity after irradiation, calculated by dividing the mean dose of cells injected into the mice before irradiation (1st column) by 10,000. The expected percentage leukaemia incidence (3rd column) was obtained from a curve relating mean dose of unirradiated leukaemia cells and the percentage of mice developing leukaemia. This curve, Fig. 2, was constructed from data of quantitative transplantation experiments reported previously (Hewitt, 1958). Thus, the expected incidences recorded in the 3rd column are those that would be obtained if the mean numbers of reproductively intact cells remaining in the mice after exposure to 1600 r radiation were in accordance with the survival curve. In spite of the small

numbers of mice used, it is seen that there is a remarkably good correlation between the expected incidence of leukaemia and the incidences observed in the present radiotherapy experiment.

The points superimposed on the curve (Fig. 2) are entered in accordance with the observed leukaemia incidences (column 4 of Table III) and the calculated mean surviving viable cell numbers (column 2 of Table III) for each irradiated group of mice. The departures of the points from the curve are well within the

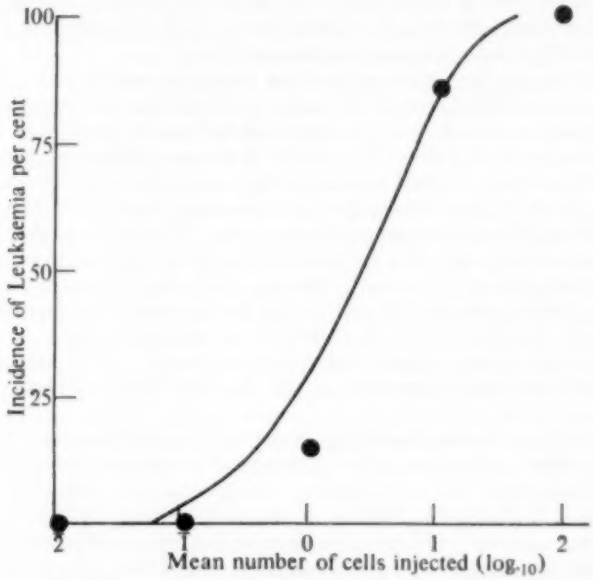


FIG. 2.—Relationship between number of CBA leukaemia cells injected intraperitoneally and proportion of injected mice developing leukaemia. The superimposed points relate to data given in Table III.

experimental error attributable to the assay method used. It is concluded that the survival curve has accurately predicted the results of radiotherapy although the survival curve data used were obtained for leukaemia cells irradiated in a quite different environment (the infiltrated livers of fully leukaemic mice).

DISCUSSION

The survival curve for CBA leukaemia cells irradiated *in vivo* (Hewitt and Wilson, 1959*a*) was originally determined in the course of attempts to explain our failure to cure leukaemic mice at an early stage of the transplanted disease by their exposure to ^{60}Co gamma whole-body radiation in doses up to 2400 r followed by treatment with isologous bone marrow. This failure contrasted with the successful treatment of a similar leukaemia by Barnes, Corp, Loutit and Neal (1956) using a method which we had duplicated in our own attempts. A

consideration of our survival curve in respect of the total leukaemia cell population estimated to be present in our mice at the time they received experimental radiotherapy revealed that the largest dose of radiation we used was not theoretically sufficient to " sterilise " a leukaemia cell population of that size. This finding suggested that the survival curve might be used to predict quite accurately the chance of a given dose of radiation being able to " cure " mice bearing an accurately measured number of leukaemia cells at the time of irradiation. We appreciated, however, that such simple application of the survival curve data as we envisaged could prove to be invalid on account of certain differences between the conditions in the radiotherapy experiments and in the survival curve experiments. These differences will be discussed further.

In the radiotherapy experiment the mice were exposed to whole-body irradiation for 16 hours at a dose rate of 1·7 r/min. : in the survival curve experiments, they were exposed to irradiation delivered in less than 30 minutes at 70–80 r/min. There is no reason to believe that such a dose-rate difference would result in different survival ratios for the exposed leukaemia cells. On the other hand, it is conceivable that in the longer exposure time some further division of the cells might ensue during the earlier phases of exposure. The total number of leukaemia cells to be " killed " would then be greater than the number of cells in a mouse at the commencement of exposure. The possible error, however, is quite insignificant, since interruption of mitosis would be expected to occur after the cells had received the first few hundred roentgens of the total dose. Moreover, even uninhibited growth of the cells during 16 hours would not be expected to result in much more than a 2-fold increase in the number of cells (Hewitt and Wilson, 1959*a*).

In the survival curve experiments, the cells which had retained their reproductive integrity after irradiation were immediately removed from the irradiated host and transplanted to normal hosts ; they were not, therefore, exposed to possible inimical influences persisting in the constitution of the irradiated host after irradiation. In the therapy experiments however, cells surviving the direct action of radiation could have been exposed to indirect effects induced in the treated hosts by the radiation. No evidence for such a persistent indirect influence was demonstrated in an experiment specifically designed to reveal it.

In the survival curve experiments, the survival ratios were determined among cells infiltrating the livers of mice with advanced leukaemia at the time of irradiation. In the radiotherapy experiments the cells were in the peritoneal cavity of otherwise normal mice during irradiation. Since the radiosensitivity of the cells heavily infiltrating the liver was shown to be compatible with their being in a moderately well oxygenated environment during irradiation (Hewitt and Wilson, 1959*b*) it is considered that cells dispersed in a small volume of fluid in the peritoneal cavity are in an environment equally well oxygenated. The environmental oxygen tension, it may be mentioned, is the only extrinsic physiological factor known to influence the radiosensitivity of the cells. In the light of present knowledge, therefore, there is no reason to suspect that the site difference would be associated with a difference of radiosensitivity expressed in terms of the mean lethal dose of radiation for the cells.

Thus, experimental enquiry failed to reveal any factor which would invalidate use of the survival curve to predict the results of radiotherapy of leukaemic mice, and simple application of the survival curve in this way showed that, in practice,

prediction was as accurate as the statistics of the experiments allowed. It appears that the rationale of the prediction procedure described would be applicable to any system in which the mean lethal dose of radiation for the effective cells and the size of the malignant cell population were known. It is, therefore, pertinent to discuss the implications of the findings reported here for other tumour-host systems, especially human cancer.

Puck, Morkovin, Marcus and Cieciura (1957) have demonstrated that a variety of malignant and non-malignant cell types of human origin, when irradiated *in vitro*, give survival curves of similar slope. It is probable, therefore, that the mean lethal dose of radiation for populations of human cells in equally well-oxygenated environments may prove to be a species characteristic only insignificantly affected by cell type or by transformation of a cell type to the malignant state. Furthermore, when suitable adjustments are made to allow for RBE differences between the qualities of radiation used in the human and murine experiments, it is clear that no significant difference can be detected between the mean lethal doses of radiation obtained for the human cell types irradiated *in vitro* and for a strain of murine leukaemia cells irradiated *in vivo* (Morkovin and Feldman, 1959, 1960; Hewitt and Wilson, 1960). Gray and Hewitt (1959, unpublished) have recently shown that the mean lethal dose of radiation for the murine leukaemia cells irradiated under well-oxygenated conditions *in vitro* is not significantly different from that previously obtained for the same leukaemia cells irradiated *in vivo* in air-breathing leukaemic mice.

Thus, whilst it is desirable to obtain mean lethal dose values for additional mouse cell types irradiated under *in vivo* and *in vitro* conditions, there are already strong indications that it might be well worthwhile to consider tumour dose requirements for the "sterilisation" of a wide variety of tumours, both in man and the mouse, in terms of a single mean lethal radiation dose (approximately 130 r, 250 kv X-rays or its biological equivalent of other radiation).

Assuming a common mean lethal dose of radiation for most human tumour cells, several further factors require close examination before this value can be used to assess tumour lethal dose requirements: estimation of the total cell population of the tumour and of the proportion that are "effective" in the sense that they can potentially form the basis of a recurrence; estimation of the proportion of "effective" cells under anoxic conditions at the time of irradiation; and the possible effect of fractionation schedules on the theoretical tumour dose requirement. In view of the very varied "radiocurability" of tumours it appears that the question of oxygen tension in the cell environment may be of decisive importance, and it is a merit of the proposed approach to the problem that the importance of this question is emphasised. It may be added that some of the known effects of fractionation of dose in the treatment of tumours may, in the event, really be due to changes in the local tissue oxygen tension brought by the reactions of the tumour bed to radiation. The intrinsic growth rate of a tumour would be expected to have a very considerable influence on the gross response of a tumour to radiation and on the length of interval between unsuccessful treatment and recurrence. It would not, however, be expected to affect the theoretical lethal tumour dose requirement, since survival curve data do not support the concept of variation of cell radiosensitivity with mitotic phase. It is clear that many of these factors would require histological examination of tumour architecture and composition by an observer specifically orientated to these special considerations

and not merely to assessment of the behaviour tendencies of the tumour derived from its histological pattern in relation to past experience. We believe that study of existing well-documented cases of radiotherapy along the lines just indicated could usefully be undertaken with the object of seeing whether the results can be adequately explained in terms of survival curve data using the mean lethal dose of radiation referred to.

SUMMARY

A survival curve for CBA mouse leukaemia cells irradiated *in vivo* in mice of the substrain of origin was described previously (Hewitt and Wilson, 1959*a*). Further experiments are here described which were undertaken with the object of examining various factors which could affect proper interpretation of that survival cure. A bioassay method was employed to show that: a heavy preponderence of admixed radiation-killed leukaemia cells did not influence the ability of small inocula of viable cells to give rise to leukaemia after transplantation; preliminary whole-body irradiation did not influence the resistance of host mice to subsequent small inocula of viable leukaemia cells. Groups of mice bearing known numbers of viable leukaemia cells were exposed to 1600 r whole-body radiation and treated with intravenous isologous bone marrow. The proportion of mice in each group which were thereby "cured" of leukaemia was strictly in accordance with prediction from the original survival curve data. This last experiment is deemed to exemplify a generally applicable thesis for the radiotherapy of malignant tumours in autologous or truly isologous systems: that the curative dose of radiation for malignant cell populations of known size irradiated *in vivo* can be prescribed from relevant survival curve data. The further problems likely to be encountered in such applications of survival curve data are discussed.

We are grateful to Mrs. J. J. Gough, B.Sc., (British Empire Cancer Campaign Research Physicist) and to Mr. N. H. Pierce (Chief Technician, Physics Department) for assistance with the irradiation procedures, and to Miss E. Blake for technical assistance with the biological work. We are indebted also to the British Empire Cancer Campaign for a whole-time research grant to one of us (H. B. H.) and for financial support for the laboratory of the other (C. W. W.).

REFERENCES

Barnes, D. W. H., Corp, M. J., Loutit, J. F. and Neal, F. E.—(1956) *Brit. med. J.*, ii, 626.

Hewitt, H. B.—(1958) *Brit. J. Cancer*, **12**, 378.

Idem and Wilson, C. W.—(1959*a*) *Ibid.*, **13**, 69.—(1959*b*) *Ibid.*, **13**, 675.—(1960) *Brit. J. Radiol.*, **33**, 198.

Klein, G.—(1959) *Cancer Res.*, **19**, 343.

Morkovin, D. and Feldman, A.—(1959) *Brit. J. Radiol.*, **32**, 282.—(1960) *Ibid.*, **33**, 197.

Puck, T. T., Morkovin, D., Marcus, P. I. and Cieciura, S. J.—(1957) *J. exp. Med.*, **106**, 485.

Reed, L. J. and Muench, H.—(1938) *Amer. J. Hyg.*, **27**, 493.

Révész, L.—(1958) *J. nat. Cancer Inst.*, **20**, 1157.

Scott, O. C. A.—(1958) 'Advances in Biological and Medical Physics', Vol. VI. New York (Academic Press Inc.), p. 121.

CARCINOGENESIS IN THE PITUITARY DWARF MOUSE. THE RESPONSE TO 2-AMINOFLUORENE

F. BIELSCHOWSKY AND MARIANNE BIELSCHOWSKY

From the Hugh Adam Cancer Research Department of the Medical School and the New Zealand Branch of the British Empire Cancer Campaign, University of Otago, Dunedin, New Zealand

Received for publication April 23, 1960

THE connective tissue elements of pituitary dwarf mice have been found to respond to a subcutaneous injection of 0·5 mg. of methylcholanthrene in the same way as those of their normal sized litter mates.

The purpose of the experiment described in this paper was to compare the susceptibility of these mice to a systemically acting carcinogenic agent, 2-aminofluorene (AF), which affects not only the parenchymal cells of a variety of organs such as liver and mammary glands, but induces also neoplastic changes in the transitional epithelium of the urinary tract.

Bickis, Estwick and Campbell (1956) did not find any spontaneous tumours in their colony of pituitary dwarf mice. Nevertheless it was felt necessary to obtain some information on the incidence of spontaneous neoplasms in old dwarfs and in the phenotypically normal heterozygotes of our stock, so that induced tumours might be distinguished from spontaneous ones. Therefore untreated animals carrying the dwarf gene were used as additional controls.

Some preliminary results of our investigation have been mentioned at the Symposium on Functional Components of Carcinogenesis held at Rehovoth in 1959.

MATERIALS AND METHODS

The origin of the mice and their maintenance have been described in a previous paper (Bielschowsky and Bielschowsky, 1959).

Whenever possible the experimental dwarfs were matched with normal sized litter mates of the same sex.

Painting of the interscapular region with a 4 per cent solution of AF in acetone was started when the mice were 8–12 weeks old. Ninety applications were given; a No. 4 brush was used for the dwarfs and a No. 6 for the normal sized animals. The total dose administered to the former was approximately 135 mg. and to the latter approximately 270 mg.

The animals were killed when a palpable tumour was present or when decline in health made it advisable, and the remainder at the 52nd week of the experiment. Their maximum age was 14½ months.

The untreated animals were kept in a separate room under the same conditions as the experimental mice. The dwarfs were killed when they were 21–25 months old. The normal sized mice were known to be heterozygotes, the majority being parents of the dwarfs treated with AF. Some of these heterozygotes had to be killed during the 13th–18th month of life because they were seriously ill, but most of them were more than 19 months old when autopsied.

The post mortem examinations of the dwarfs were carried out under a magnifying glass. The histological methods used were those previously described (Bielschowsky and Bielschowsky, 1959).

RESULTS

The average weight of the dwarfs at the start of the experiment was 7 g. and 8·5 g. at the time when treatment with AF was stopped. The corresponding values for the normal sized mice were 23 and 26 g. Since the amount of AF given to the dwarfs was approximately 50 per cent of that given to their phenotypically normal litter mates the former received, per gram body weight, more than the latter. Of the 55 dwarfs treated with the carcinogen, 39 survived for at least 29 weeks, the time when the first tumour, a cancer of the bladder, was found. All other tumours observed in this group occurred in animals killed in the 39th–52nd week of the experiment.

In the normal sized litter mates the earliest tumour, also a cancer of the bladder, was found in the 34th week. The average age of these mice at death was 4½ weeks less than that of the dwarfs.

Table I shows the neoplastic lesions found at autopsy in untreated controls (Groups I and II) and Table II those seen in the mice treated with AF (Groups III and IV). A comparison of the tumour incidence in the four groups reveals differences between the dwarfs (Groups I and III) and the phenotypically normal mice (Groups II and IV) in respect to both spontaneous and induced tumours. For instance, in the 41 normal sized mice treated with AF (Group III) 32 hepatomas were found, but only 13 liver tumours in the 39 dwarfs of group IV. There was therefore a highly significant association of normal body size with hepatoma formation ($P < 0{\cdot}001$). In the dwarfs both sexes were equally affected. In group III however there was a prevalence of hepatomas in the females, 90 per cent of

TABLE I.—*Neoplastic Lesions Found at Autopsy in Untreated Controls*

	Control animals	
	Group I	Group II
	Neoplastic lesions found in	
	Normal sized untreated	Dwarfs untreated
Total number of animals	28 (♂ 14, ♀ 14)	37 (♂ 17, ♀ 20)
Age in months	13–25	21½–25½
Animals with tumours in—		
Liver	—	3
Lung	11	—
Duodenum	4	1
Breast	2	—
Pituitary	2	—
Ovary	1	2
Lymph glands	1	—

which developed tumours of the liver, whereas the incidence in the males of this group was 65 per cent.

The first palpable hepatomas occurred in 2 normal sized females during the 37th week of the expriment. Two weeks later a male dwarf of group IV was found to have a liver tumour with a diameter of 3 mm. Three blocks were taken from

TABLE II.—*Neoplastic Lesions Seen in Mice Treated with AF*

	Experimental animals	
	Group III	Group IV
	Neoplastic lesions found in	
	Normal sized treated with A.F.	Dwarfs treated with A.F.
Total number of animals	41 (♂ 20, ♀ 21)	39 (♂ 17, ♀ 22)
Duration of experiment (weeks)	34–52	29–52
Animals with tumours in—		
Liver	32 (♂ 13, ♀ 19)	13 (♂ 7, ♀ 6)
Lung	5	—
Pyloric region	12	3
Breast	8	—
Bladder	4	4
Kidney	1	—
Ovary	1	—
Uterus	1	—
Gall bladder	2	—

those livers of mice of groups III and IV which at post mortem appeared normal. Histological investigation failed to reveal neoplastic changes in those of normal sized mice but in 2 of the dwarfs minute lesions were seen. They were formed by the same type of cell depicted in Fig. 1, a photomicrograph of a macroscopically recognizable hepatoma. This indicates that more hepatomas might have developed in the dwarfs treated with AF had these animals been observed for a longer period. Histologically there were no essential differences between the liver tumours found in groups III and IV, but in the latter, occasionablly foci of regressing neoplastic lesions (Fig. 3) were seen in livers which at the same time contained progressing tumours (Fig. 1 and 2). Only once, in a dwarf, were deposits of hepatoma cells detected in the lungs (Fig. 4).

Spontaneous liver tumours were not found in untreated heterozygotes, but 3 of the dwarfs of group II had one or more macroscopically recognizable nodules in the liver. Only in one instance did a neoplastic lesion resemble those seen in the liver of the experimental animals (Fig. 5). In the 2 others the histological picture was of a more benign nature. One old dwarf had a single yellowish coloured nodule composed of pale plant-like cells (Fig. 6), the other had 5 distinct nodules the cells of which differed only slightly, by the basophilia of the cytoplasm, from the surrounding liver cells. In this connection it seems worth mentioning that areas of necrosis were not infrequent in the livers of old untreated dwarfs, but amyloid present in some livers of untreated heterozygotes was never seen.

Benign papillomas of the gall bladder (Fig. 7) occurred in 2 of the normal sized mice treated with AF.

No breast tumours developed in the dwarfs of groups II or IV, but 2 spontaneous mammary cancers were seen in the untreated heterozygotes and 8 occurred in the normal sized mice treated with AF. The spontaneous tumours were typical alveolar carcinomas and all the latter contained acanthotic areas.

There was little difference between the dwarfs and their litter mates in the response of the transitional epithelium to AF. In 4 animals of each group macroscopically recognizable bladder tumours were seen. Three were classified as malignant because the muscular layer of the bladder was deeply invaded. One such cancer occurred in a dwarf. In addition, there was one carcinoma of the

pelvis in an animal of group III. No spontaneous tumours of the urinary tract were found in the untreated mice, but benign papillomas of the duodenum occurred in a few mice of groups I and II. The incidence of these lesions was considerably higher in the animals treated with AF. In the heterozygotes of group III 12 tumours of the intestinal tract, situated mainly on either side of the pylorus, were found. Two of these tumours had invaded the muscular layer (Fig. 8). On the whole they were considerably larger than the spontaneous ones and the same holds true for the three benign papillomas found in the dwarfs treated with AF (Fig. 9 and 10).

Tumours of the lung were not found in any of the dwarfs of either group II or IV although this was the most common spontaneous tumour in the heterozygotes, 40 per cent of which had single pulmonary adenomas. Of the normal sized mice treated with AF, 5 developed benign lung tumours. In 3 of these animals they were multiple but were of a smaller size than those observed in the much older mice of group I.

All the ovarian tumours found, including the one seen in an animal of group III, are considered to be spontaneous benign neoplasms. Three of them were granulosa cell tumours and the fourth appeared to arise from the cells of the ovarian stroma.

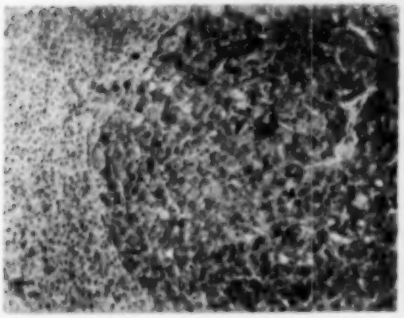
DISCUSSION

In the rat ablation of thyroid (Bielschowsky and Hall, 1953) or pituitary (O'Neal, Hoffman, Dodge and Griffin, 1958) prevents the development of hepatomas induced by AF or related compounds, but the liver of the pituitary dwarf mouse is susceptible to the action of this aromatic amine. In spite of the absence of growth hormone and in spite of an extremely low level of thyroid function, the liver cells of the dwarf react to the carcinogen qualitatively in a similar manner as those of normal mice or intact rats. The development of hepatomas is slowed down, but not inhibited. In conjunction with these findings the occurrence of spontaneous liver tumours in 2-year-old dwarfs is of considerable interest and stresses the fact that in pituitary dwarf mice, at least, a multiple hormone deficiency does not prevent neoplastic growth in the liver.

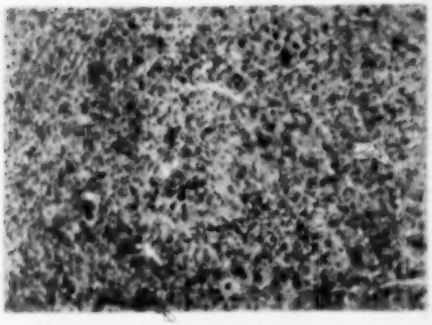
The failure of the female dwarfs to develop breast tumours is easily explained by the virtual absence of mammary gland tissue ; they do not even have nipples. Although gonadotrophs are present in the pituitary of the dwarf mouse there is hardly any indication of oestrogen secretion by the ovaries. Uterus and vagina always showed the picture of sexual immaturity ; also in our material there was

EXPLANATION OF PLATES

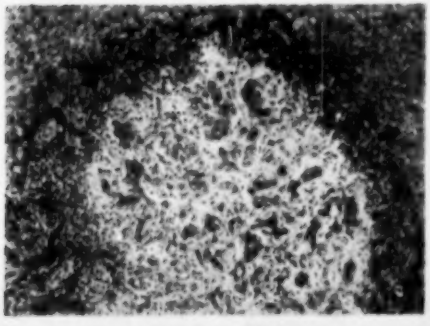
FIG. 1.—Hepatoma formed by large cells with eosinophilic cytoplasm from a dwarf of group IV. H. and E. ×80.
FIG. 2.—Another hepatoma from the same dwarf as Fig. 1. H. and E. ×80.
FIG. 3.—A regressing neoplastic lesion—same dwarf as Fig. 1 and 2. H. and E. ×80.
FIG. 4.—Hepatoma cells in lung of a dwarf of group IV. H. and E. ×80.
FIG. 5.—Spontaneous hepatoma from a dwarf of group II. H. and E. ×80.
FIG. 6.—Benign nodule from dwarf of group II. H. and E. ×80.
FIG. 7.—Benign tumour of the gall bladder from a normal sized mouse of group III. H. and E. ×80.
FIG. 8.—Adenocarcinoma of small intestine from a normal sized mouse of group III. H. and E. ×20.
FIG. 9.—Papilloma of small intestine from a dwarf of group IV. H. and E. ×20.
FIG. 10.—Papilloma of duodenum from another dwarf of group IV. H. and E. ×20.



1

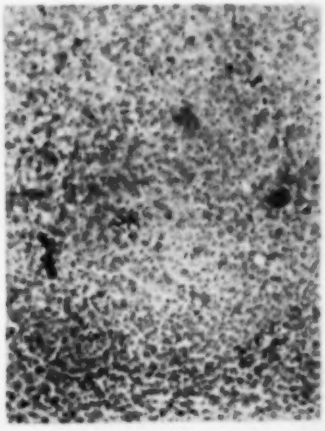


2



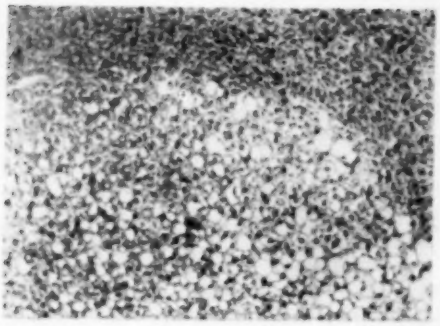
3

4

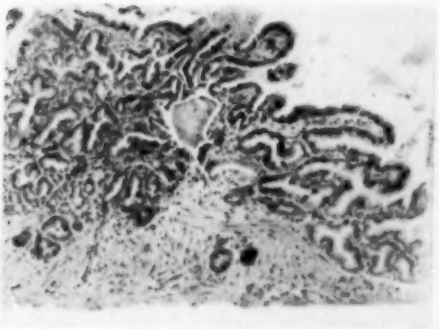


5

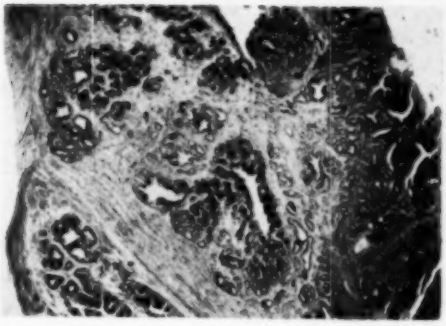
Bielschowsky and Bielschowsky.



6

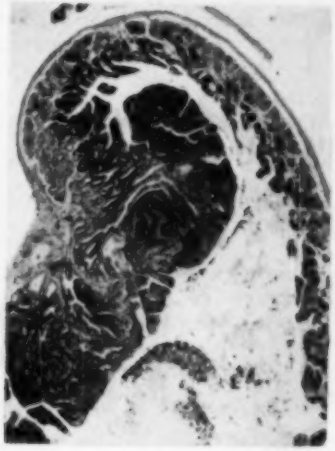


7



8

9



10

Bielschowsky and Bielschowsky.

never any sign of luteinization in the ovaries. Therefore the four hormones which normally stimulate the mammary glands, oestrogen, progesterone, prolactin and growth hormone, if present at all, were secreted in only extremely low quantities. In these animals the only evidence for gonadotrophic action was the size of the follicles. Some were larger than those seen in the gonads of females hypophysectomized when sexually immature, but they never reached the size and appearance of mature follicles. Nevertheless in 2 ovaries of old untreated dwarfs non-functional neoplastic lesions were found. In this context it might be mentioned that in the male dwarfs evidence for gonadotrophic stimulation was more obvious. The testes of some dwarfs had descended into the scrotum and in such glands sperm was produced. However, stimulated secondary sex organs were rarely seen in a male dwarf.

In view of the occurrence of spontaneous benign papillomas of the small intestine in both groups of untreated animals it seems doubtful that the similar tumours observed in mice of groups III and IV were induced by AF. The higher incidence of these neoplasms, the larger size and the invasiveness of some of them suggests that the carcinogen accelerated their development. In the dwarfs this process seems to progress more slowly than in the normal sized animals ($P = 0{\cdot}013$).

The absence of lung tumours in the dwarfs is surprising because in the normal sized animals these neoplasms were far from infrequent. Of 17 untreated heterozygotes, older than 20 months, 9 had pulmonary adenomas. It is well established that the genetic constitution determines the susceptibility of mice to the development of spontaneous as well as induced adenomas of the lung (Heston, 1942), whereas, as far as we are aware, there is no evidence for hormones playing an important role in their pathogenesis. Still, in view of the close relationship of the experimental mice of groups III and IV which were litter mates, and the untreated heterozygotes of group I which were their parents, it seems difficult to believe that non-systemic genetic factors could account for the difference in incidence.

Further experiments are under way to test whether or not it is possible to obtain lung tumours in the pituitary dwarf mice of our stock with the aid of other carcinogenic agents.

SUMMARY

The susceptibility of pituitary dwarf mice to 2-aminofluorene was compared with that of their normal sized litter mates.

In the dwarfs the transitional epithelium of the bladder was found to be as sensitive as that of the phenotypically normal animals, but, in the former, liver cells and the epithelium of the small intestine reacted in a significantly slower manner to the carcinogen.

No tumours of the breast or lungs were found in the dwarfs whether treated or untreated. In the closely related normal sized animals these organs exhibited benign and malignant neoplasms.

REFERENCES

Bickis, I., Estwick, R. R. and Campbell, J. S.—(1956) *Cancer*, **9**, 763.
Bielschowsky, F. and Bielschowsky, M.—(1959) *Brit. J. Cancer*, **13**, 302.
Idem and Hall, W. H.—(1953) *Ibid.*, **7**, 358.
Heston, W. E.—(1942) *J. nat. Cancer Inst.*, **3**, 79.
O'Neal, M. A., Hoffman, H. E., Dodge, B. G. and Griffin, A. C.—(1958) *Ibid.*, **21**, 1161.

SERIAL IRRADIATION OF MOUSE TUMOURS: SOME CHANGES IN HISTOLOGY AND CYTOLOGY

A. E. G. PEARSON

From the Department of Experimental Pathology, Mount Vernon Hospital, Northwood, Middlesex

Received for publication April 23, 1960

THE method of serially irradiating mouse tumours by transplantation into fresh hosts between each irradiation, enables histological changes to be observed divorced from the direct and indirect local effects of irradiation on the tumour bed. Snellman (1935), employing this method, observed an increase in collagen in serially irradiated Jensen sarcoma in rats and interpreted this as indicating an increase in the degree of tumour differentiation. He also demonstrated a decrease in radiosensitivity in this irradiated line. It can be postulated that the increase in degree of differentiation may have occurred by selection from a mixed population originally containing more anaplastic cells, thereby assuming them to possess a greater radiosensitivity, or by transformation to a differentiating condition. The correlation between radiosensitivity and these observations is obscure. Glucksmann (1948) favoured the opinion that the radiocurable tumours were distinguished by their capacity for increased differentiation after radiation; this is at variance with the observations of Snellman.

No changes in radiosensitivity at the lethal dose level were found by Pearson (1959*a*) in four serially irradiated mouse tumour lines. This communication reports some changes in histology and cytology observed in these lines.

MATERIALS AND METHODS

The methods employed in establishing serially irradiated lines of sarcoma 37 and two homologous tumours in RIII strain mice, have been reported previously (Pearson, 1959*a*). One irradiated line of sarcoma 37 received tumour sub-lethal doses of 3000 r at each stage ("B" line) and one received half-lethal doses of 2000 r ("D" line). Tumour sub-lethal doses were administered at each stage to the homologous spindle-celled sarcoma BP1 ("F" line) and mammary adenocarcinoma MV212 ("G" line). Tumour material for histological study was obtained from transplanted post-irradiation tumours from each irradiation stage and also from untreated sub-line passages of the 12th stage of the sarcoma 37 "B" line.

The tumour material was treated with Susa fixative for 2 to 3 hours and embedded in paraffin wax by chloroform substitution from alcohol. Sections were cut at 6 μ and stained in haematoxylin and eosin.

RESULTS

No marked histological differences were apparent between tumours of the spindle-celled sarcoma BP1 (Fig. 2 and 3) and adenocarcinoma MV212 and their respective irradiated lines "F" and "G". Small regions in "G" line tumours

after three serial irradiations exhibited a decreased basophilia and a reduced tendency to form acini, these areas were absent in control MV212 tumours (Fig. 4 and 5).

The tendency to produce a liquid necrotic centre by sarcoma BP1 tumours was greatly reduced in its irradiated line. Control tumours commenced to form a necrotic centre on attainment of an area, measured externally, of about 60 sq. mm. This necrosis was usually delayed in " F " line tumours until an area of 90–100 sq. mm. was reached.

Histological variations in the cortical region were observed between sarcoma 37 and " B " line tumours. An extensive cortical region, consisting of invasive loosely connected tumour cells was present in controls (Fig. 6). " B " line tumours at the 12th irradiation stage possessed no comparable cortex, the edge of the tumours consisted of packed cells and a thinner capsule (Fig. 7). Even in regions where the connective tissue capsule had greater depth no invasion by tumour cells occurred. These histological differences were observed until the 17th irradiation stage, when the series was discontinued ; were apparent at the 7th stage and were still present at the 32nd sub-line passage (without further irradiation) of the 12th stage. The 4th to 6th stages exhibited an intermediate condition.

No differences were observed between sarcoma 37 and its irradiated " D " line, after 10 serial half-lethal dose irradiations.

Studies on the reticulin distribution in sarcoma 37 and B12 showed a sparse loose network in the centres of both tumours, reported by Mackenzie (1958) to be associated with anaplasia. The transition between this loose network and the denser, more regular concentric patterns in the capsule was gradual in control and abrupt in B12 tumours (Fig. 8 and 9).

Cytological comparisons between sarcoma 37 cells and those of its irradiated " B " line revealed marked changes in nuclear morphology. Nuclei from control tumour cells were usually kidney-shaped or irregular in outline ; the chromatin, stained with haematoxylin, comprising several small discrete bodies with a diffuse staining of the nuclear sap (Fig. 10). Most cells of the B12 irradiation stage possessed nuclei which had prominent usually single nucleoli, few chromatin bodies and a less intensely stained nuclear sap (Fig. 11). These latter nuclei conformed closely to the description by Caspersson and Santesson (1942) of " B " cell nuclei, the control sarcoma 37 cells conformed to the " A " type of these authors. This nomenclature is employed hereafter.

In order to determine whether the large staining bodies in " B " cell nuclei were true nucleoli (plasmosomes) comprised of ribose nucleic acid (RNA) or false nucleoli (chromocentres) comprised of deoxyribonucleic acid (DNA), sections were stained by the " azan " method of Heidenhain. These large chromatin bodies stained red by this method and were therefore true nucleoli ; the bulk of chromatin in " A " cell nuclei stained blue but usually 2 to 4 small nucleoli were also present. Prominent chromocentres were not observed in " B " cell nuclei. These observations were confirmed by a negative reaction of the large " B " cell nucleoli to Feulgen stain.

Control tumours were found to contain some cells of the " B " type and irradiated line tumours contained " A " type cells. Proportional counts were therefore made of these cell types in tumours from all the irradiation stages (B1 to B16), the 26th sub-line passage of the 12th stage (B12/26) and from the 10th stage of the irradiated " D " line (D10). Five high power ($\times 675$) fields each were

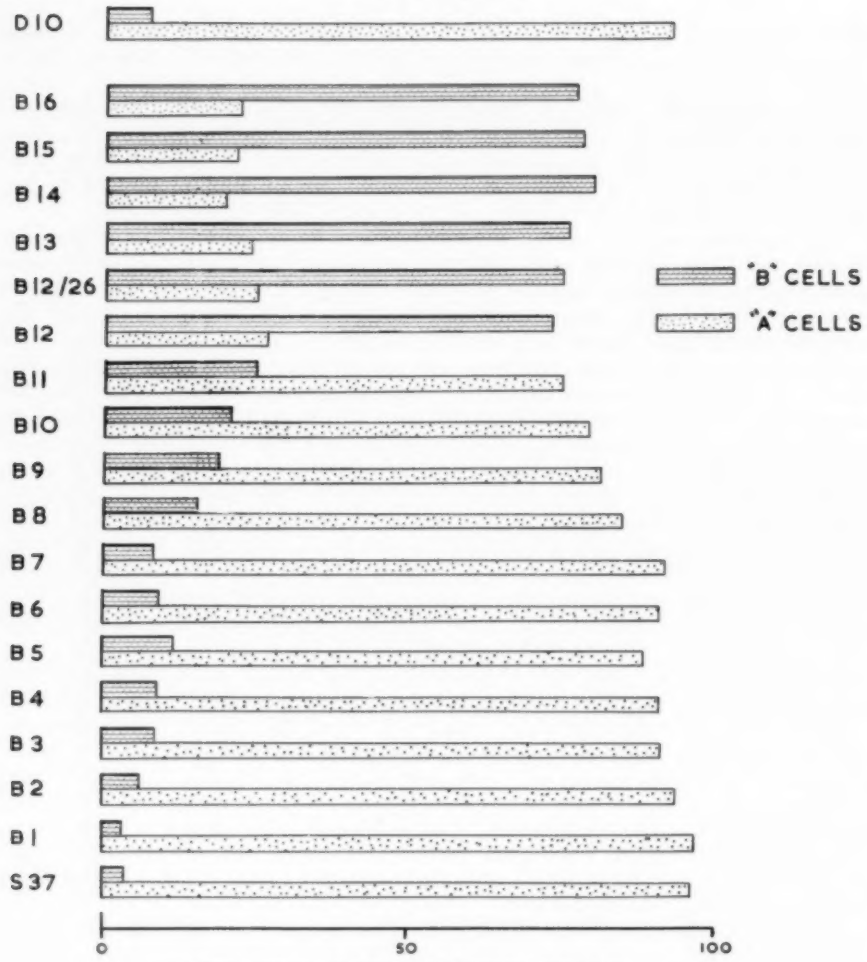
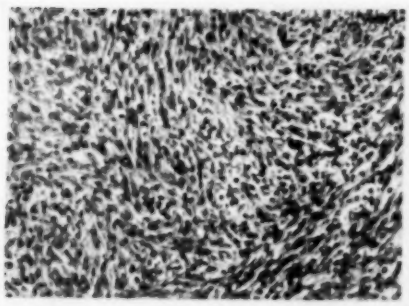


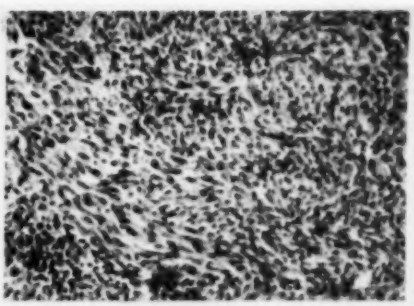
FIG. 1.—Proportions of " A " and " B " cells in sarcoma 37, serial irradiation stages of its " B " line (B1–B16), 26th sub-line passage without further irradiation from the 12th stage (B12–26) and the 10th irradiation stage of its " D " line receiving half-lethal dises at each stage (D10).

EXPLANATION OF PLATES

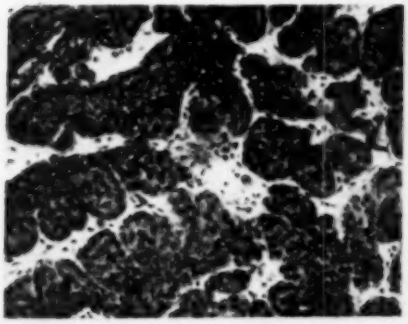
FIG. 2.—Sarcoma BP1. ×100.
FIG. 3.—Sarcoma BP1 irradiated line " F ", 7th stage. ×100.
FIG. 4.—Mammary adenocarcinoma MV212. ×100.
FIG. 5.—Mammary adenocarcinoma MV212 irradiated line " G ", 10th stage, showing region of increased anaplasia. ×100.
FIG. 6.—Sarcoma 37, cortical region. ×100.
FIG. 7.—Sarcoma 37 irradiated line " B ", 12th stage, cortical region, showing reduced capsule and loss of invasive properties. ×100.
FIG. 8.—Sarcoma 37, cortical region, distribution of reticulin. ×120.
FIG. 9.—Sarcoma 37 irradiated line " B ", 12th stage, cortical region, distribution of reticulin. ×120.
FIG. 10.—Sarcoma 37 showing typical " A " cells. ×960.
FIG. 11.—Sarcoma 37 irradiated line " B ", 12th stage, showing typical " B " cells. ×960.



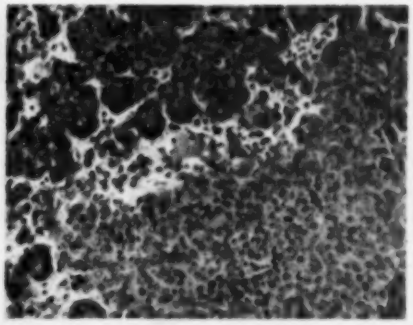
2



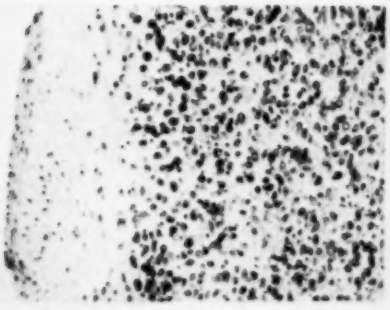
3



4

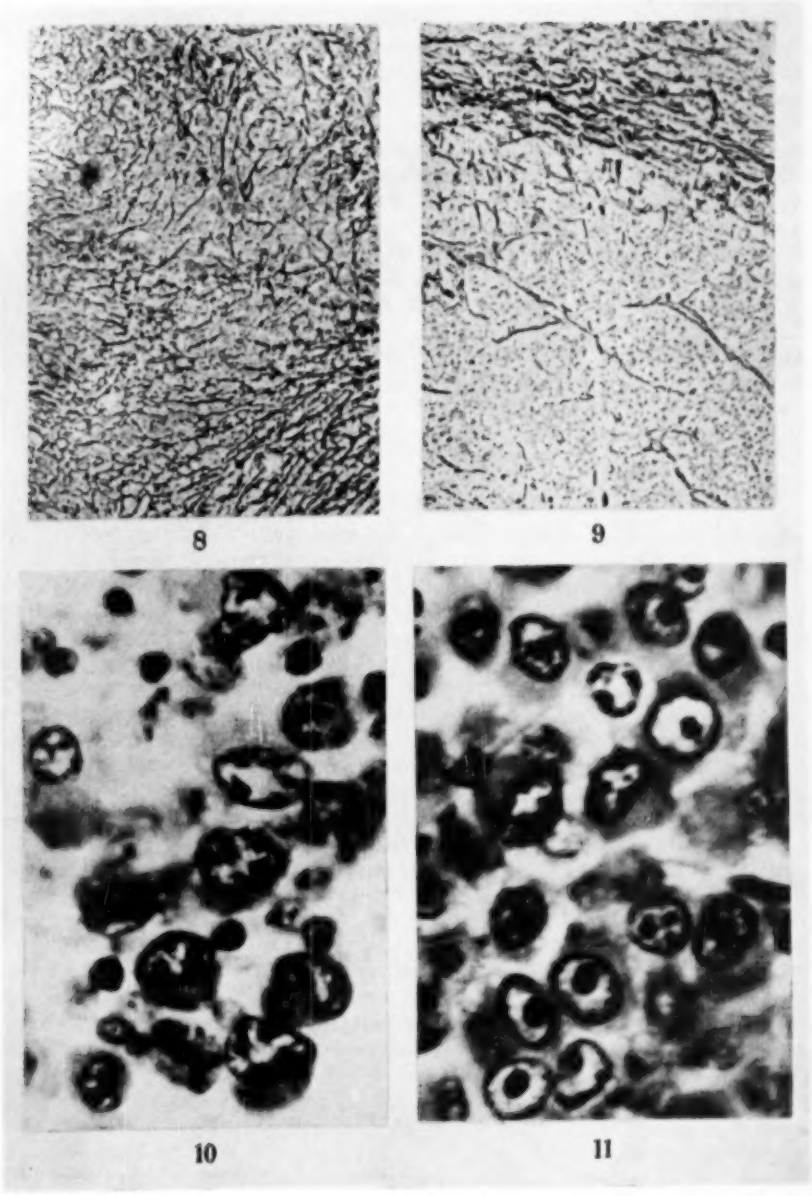


5



6

7



8 9

10 11

Pearson.

counted from cortical and central regions of tumours from each irradiation stage. No consistent marked differences in proportionality were detected between the two regions and the data were therefore combined, resulting in a total count of 2000 to 2590 cells for each stage. Control data were obtained from tumour material fixed at intervals covering the period taken to establish the irradiated line. No significant differences were found in this control material.

The results of these counts are presented in Fig. 1. It can be seen that the irradiation treatments favoured an early uniform increase in the proportion of " B " cells up to the 11th stage. At this stage 25 per cent of the total cells were of the " B " type, but after the next irradiation treatment this proportion had increased to about 75 per cent and remained near this level at subsequent stages. Sub-line passage (B12/26) did not affect this proportion and it would appear that a stable condition had been reached.

The proportion of " B " cells in the " D " irradiated line at the 10th stage (Fig. 1, D10) was 7·3 per cent compared with 3·8 per cent in control tumours. The chi-square test of significance applied to this data gave a probability of 4 per cent ; the increase in " B " cells was therefore probably significant.

DISCUSSION

No marked changes in the degree of differentiation were observed after serial irradiation—even in the less anaplastic tumours. A tendency in the irradiated " G " line of MV212 towards an increase in anaplasia is opposed to the observations of Snellman (1935). Since no changes in radiosensitivity at the lethal level were observed (Pearson, 1959*a*), in this instance radiocurability cannot be correlated with the degree of differentiation.

The change in behaviour of sarcoma 37 " B " line cells in the cortical region may have been linked with the relative increase in " B " cells, although the same histological change was present at the 7th stage when the proportion of " B " cells had only risen to 8·3 per cent. It may be postulated that these cells were unable to invade the adjacent tissue ; however tissue cultures from both tumour lines showed both cells types in the form of actively migrating spindle-shaped cells. Twenty-five per cent of the cells at the 12th stage were of the " A " type and it would be reasonable to expect some of these cells to show invasive properties, as this condition was not observed, both cell types must have been altered by the irradiations.

Caspersson and Santesson (1942) postulated that the " B " cell type was a degenerate form of " A " cell ; this was based on evidence that " B " cells had a lower protein content and occupied a more central position within the tumour. No zonal variations were found in the irradiated line and if the " B " cells represented a degenerate form a reduced mitotic coefficient would be expected owing to their increased proportion. Mitotic coefficients for (*a*) control and irradiated line tumours of (*b*) comparable age and (*c*) comparable size, the latter tumour line having a reduced growth rate (Pearson, 1959*b*), were 1·75, 1·74 and 1·66 respectively. Total cells counted were between 14,600 and 18,000 in each group and the differences in mitotic proportions between the samples lay within the standard error. If the " B " cells were a post-mitotic degenerate form a substantial increase in the mitotic rate of " A " cells in the irradiated line would be necessary to account for the comparable overall rate.

The glassy-cell type of mixed carcinoma of the cervix, described by Glucksmann and Cherry (1956), appears to possess a nuclear norphology comparable to the " B " cells. These authors stated that this type of adenoacanthoma was comparatively refractory to irradiation. Gusberg *et al.* (1954 and 1956) suggested that the proportions of " A " and " B " cells in cervical cancer could be correlated with the clinical results from irradiation, a rise in the " B " cell proportion indicating a favourable result ; the assumption was made that the " B " cells were a degenerate form. The radiocurability of sarcoma 37 however was not significantly altered in its irradiated line, in spite of the considerable increase in " B " cells. It would appear therefore that cells of this nuclear morphology may not necessarily have comparable biological properties.

Measurements of DNA in control and irradiated line sarcoma 37 tumours (Pearson and Atkin, 1960) showed that the tetraploid mode in control tumours was replaced in the irradiated line by a mode corresponding to a triploid condition. Allowing for errors in the selection of classes in the DNA histograms and assuming the morphology of hexaploid and triploid nuclei to be comparable, the proportions of " A " and " B " cells in the two tumour lines are comparable to those of $4n$, $8n$ and $3n$, $6n$ cells estimated by DNA measurement. It would appear that a possible correlation exists between ploidy and nuclear morphology in this case.

It is of interest that in the sarcoma 37 experiments the serial application of irradiation produced permanent observable changes, not dependent on the continuation of the stress, which did not affect the radiosensitivity of the tumour. This suggests that a selection or change by irradiation of certain cell properties can occur without affecting the fundamental radiosensitive features directly concerned with cell reproducibility or viability.

SUMMARY

1. Histological and cytological comparisons have been made on parent and serially irradiated lines of sarcoma 37 and two homologous tumours in RIII strain inbred mice.

2. The two homologous tumours showed no marked changes after serial irradiation.

3. Regions of increased anaplasia were apparent in the adenocarcinoma MV212 after serial irradiations.

4. Histological changes were apparent in the cortex of sarcoma 37 tumours subjected to sub-lethal doses at each irradiation stage.

5. In this same line a proportional increase occurred in certain cells having a characteristic nuclear morphology.

6. Both these changes appeared to be progressive during the establishment of the irradiated line and were not altered by sub-line passage without further irradiation treatment.

7. The significance of these changes is discussed in relation to radiosensitivity, cell degeneration and invasive properties.

8. A correlation is suggested between nuclear morphology and ploidy.

The expenses of this research were defrayed from a block grant by the British Empire Cancer Campaign.

REFERENCES

CASPERSSON, T. AND SANTESSON, L.—(1942) *Acta radiol., Stockh.*, Suppl. 46.
GLUCKSMANN, A.—(1948) *Brit. J. Radiol.*, **21**, 559.
Idem AND CHERRY, C. P.—(1956) *Cancer*, **9**, 971.
GUSBERG, S. B., TOVELL, H. M. M., EMERSON, R. AND ALLINA, H.—(1954) *Amer. J. Obstet. Gynec.*, **68**, 1464.
Idem, TOVELL, H. M. M., LONG, M. AND HILL, J. C.—(1956) *Ann. N.Y. Acad. Sci.*, **63**, 1447.
MACKENZIE, D. H.—(1958) *Brit. J. Cancer*, **12**, 14.
PEARSON, A. E. G.—(1959*a*) *Ibid.*, **13**, 477.—(1959*b*) *Ibid.*, **13**, 699.
Idem AND ATKIN, N. B.—(1960) *Nature, Lond.*, **186**, 647.
SNELLMAN, B.—(1935) *Acta radiol., Stockh.*, **16**, 545.

THE NEOPLASTIC POTENTIALITIES OF MOUSE THYROID UNDER EXTREME STIMULATION

M. S. ISRAEL AND I. ROSEMARY ELLIS

From the Department of Pathology, Royal College of Surgeons of England, Lincoln's Inn Fields, London, W.C.2

Received for publication April 20, 1960

HYPERPLASIA of the thyroid gland will follow any procedure leading to thyroid hormone deficiency provided the anterior pituitary is intact. This has been demonstrated in rats and mice treated with thiouracil (Astwood, Sullivan, Bissell and Tyslowitz, 1943; Gorbman, 1947) and also after the use of a low iodine diet (Remington, 1937; Axelrad and Leblond, 1952). Attempts to produce thyroid neoplasia by these means have been relatively successful in rats (Hall and Bielschowsky, 1949; Axelrad and Leblond, 1955), but the mouse responds much less readily; indeed serial transplantations of such thyroid tissue through several generations of mice have usually been required to establish clear evidence of malignancy.

In the present experiment the neoplastic potentialities of mouse thyroid gland under the stimulation of extreme induced thyroxin deficiency has been assessed by subjecting a group of mice to a combination of a thiouracil derivative and a low iodine diet.

MATERIALS AND METHODS

One hundred 4-month-old C57 mice, 50 males and 50 females, were segregated by sex in metal cages, 6 to 7 per cage. They were then divided into 3 groups:

Group A—the control series, consisting of 12 males and 13 females, was given a stock 41B diet and tap water.

Group B—consisting of 25 males and 25 females, was given the stock diet and distilled drinking water with methylthiouracil added.

Group C—consisting of 13 males and 12 females, was fed on a special low iodine diet in addition to the distilled water with methylthiouracil added.

Methylthiouracil solution.—A stock 5 per cent solution was made by dissolving 10 g. methylthiouracil in 40 ml. of 2N-sodium hydroxide and diluting this to 200 ml. with distilled water. The use of sodium hydroxide was necessary because of the poor solubility of methylthiouracil in water. This stock solution was diluted with distilled water to give a final concentration of 0·05 per cent methylthiouracil. This was given as drinking water to Groups B and C.

Low iodine diet.—This was recommended by Dr. R. Pitt-Rivers (1957, personal communication), of the National Institute for Medical Research. It consisted of:

Wholemeal flour	.	1000 parts
Wheat gluten	.	660 ,,
Iron calcium flour	.	200 ,,
Drodisol yeast	.	140 ,,

The iron calcium flour consisted of:

Calcium hydrogen phosphate ($CaHPO_4$) . .	40 parts
Hydrated ferrous sulphate ($FeSO_4.7H_2O$) .	2 ,,
Wholemeal flour	100 ,,

The mice were killed after 480 days and necropsies were performed. The thyroid glands were removed intact, together with the enveloped larynx and first ring of the trachea, all of which was weighed together. The thyroids were not dissected free of the trachea lest histological evidence of invasion might be lost. The thyroid glands and both lungs were fixed in 10 per cent formol saline. Paraffin sections were cut and stained with Erhlich's haematoxylin and eosin.

The pituitary glands were carefully removed for further studies, which will be reported later.

RESULTS

Group A.—The mice were in good condition and their weights ranged from 26 to 32 g. The thyroid glands were small and uniform in consistency, and the combined weights of thyroid and attached trachea ranged from 12 to 20 mg.

The histological appearance was one of regularly arranged large round acini full of homogeneous, brightly eosinophilic colloid. The acini varied in size but they were all lined by a uniform low cuboidal epithelium (Fig. 1). In none was there any evidence of hyperplasia.

Group B.—The mice were in good condition and their weights ranged from 22 to 30 g. The thyroid glands were greatly enlarged and nodularity was usually obvious. Adhesion to neighbouring structures was not encountered. The combined weights of thyroid and attached trachea ranged from 38 to 87 mg.

The histological appearance was one of profound hyperplasia of variegated pattern, though of fundamentally stereotyped structure. The basic feature was acinar hyperplasia—groups of irregularly enlarged acini lined by an exuberant low columnar epithelium. Some acini were empty, others contained pale-staining colloid and a few were greatly distended with brightly eosinophilic colloid. The hyperplasia was diffuse in extent, and in many areas there was a pronounced invagination of papillary epithelial processes into the larger acini (Fig. 2). This change merged imperceptibly into the most characteristic feature of all, papillary hyperplasia (Fig. 3) which was present to greater or lesser degree in all the treated animals. It consisted of the invagination of copious strands of hyperplastic epithelium supported on thin cores of vascular connective tissue into large cystic spaces, most of which were empty, though a few contained colloid (Fig. 4). The epithelial ingrowth was so intricate that the acini were compressed into narrow, sinuous, cleft-like channels. Demarcation of papillary areas into circumscribed nodules was common, and an appearance close to that of true papillary adenomata was sometimes encountered in the most hyperplastic glands (Fig. 5). The cells, however, were uniform in appearance, though larger and paler than those of normal acini. The nuclear pattern was regular and mitotic figures were extremely rare (Fig. 6). In the most hyperplastic glands some of the cells were very large and had massive nuclei (Fig. 7).

A less common variant was solid cell hyperplasia, in which clumps of pale columnar cells with little tendency to the formation of central lumina appeared in circumscribed nodules (Fig. 8).

In no case was there evidence either of infiltration into the trachea or the surrounding muscles or of pulmonary metastases.

There were no other conspicuous post-mortem findings except in 2 female mice: one had a greatly enlarged spleen due to intense lymphoid hyperplasia and the other a large, blood-filled simple ovarian cyst. The pituitary glands were not conspicuously enlarged in any of this group.

There appeared to be slightly greater focal nodular papillary hyperplasia of the thyroids in the females than in the males, but the difference was not outstanding.

Group C.—One mouse died naturally 4 days before the end of the experiment. The remainder were in poor condition: their coats were dull and lustreless and they were undernourished. This was reflected in their weights, which ranged from 15 to 21 g.

Great thyroid enlargement was again present, but the glands did not differ markedly from those of Group B, with the exception of the animal that died spontaneously, in which the gland was replaced by a large cancer which had infiltrated surrounding structures and caused death. The combined weight of this mass and the trachea was 147 mg.; in the others the weights ranged from 40 to 90 mg.

The histological picture of profound papillary hyperplasia was augmented by the presence of papillary adenomata in 11 of the 25 glands. These consisted of circumscribed collections of proliferating cells arranged in a papillary pattern (Fig. 9). They closely resembled exuberant papillary hyperplasia, but the crowded, irregular arrangement of the cells and the distinctive cellular morphology indicated focal neoplastic transformation. The cells were larger, more polygonal, their copious cytoplasm was much more basophilic and their large, darkly-staining nuclei were more irregular in shape and size than those of the neighbouring hyperplastic areas (Fig. 10). Mitotic figures were scanty. Some adenomata were very large and were composed of sheets of cells arranged in broad papillae (Fig. 11). In these the possibility of early neoplastic change could not be excluded, but there was no evidence of local infiltration.

In the mouse with macroscopic cancer histological examination showed complete replacement of the thyroid by a poorly-differentiated adenocarcinoma whose cells were arranged in large papillae in some areas and in irregular acini in others. Colloid was not found in these acini (Fig. 12). The lungs of this mouse contained many metastases throughout their substance and subpleurally; these were rather better differentiated than the primary tumour, for a few acini contained colloid (Fig. 13). In the other animals no pulmonary deposits were present.

Pituitary enlargement was conspicuous in this group, and in 8 mice large pituitary adenomata were present. One female mouse had a large anaplastic carcinoma of the caecum.

Of the 11 thyroid adenomata 8 occurred in females and 3 in males. The carcinoma, however, occurred in a male mouse.

DISCUSSION

Both thiouracil administration and a low iodine diet act by interfering with thyroxin synthesis. A deficiency of circulating thyroid hormone causes hyper-

plasia of those cells of the pituitary responsible for the secretion of thyrotrophic hormone (Russfield, 1955). Frank adenomata of these cells have been produced experimentally in mice given large doses of radioactive iodine (Burt, Landing and Sommers, 1954). The thyrotrophic hormone induces hyperplasia of the thyroid gland.

It is of great interest to determine whether this hyperplasia can proceed to of state of neoplasia, because such tumours would have been induced by an intrinsic alteration of internal environment following a derangement of hormone synthesis rather than by the action of some extrinsic carcinogen.

In the rat such neoplasia has been produced quite easily (Bielschowsky, 1955). Indeed Hall and Bielschowsky (1949) demonstrated that, although 2-acetylaminofluorene potentiated the neoplastic effect of thiouracil on rat thyroid during the first year of administration, malignant tumours developed quite as readily without the addition of the carcinogen after 18 months.

In the mouse it is much more difficult to produce thyroid tumours. It is clear that an exuberant pattern of hyperplasia is characteristic, and even the presence of discrete adenomata, valuable indications of progressive cellular proliferation, will not suffice as incontrovertible evidence of neoplastic change. Only definite infiltration of surrounding structures and the presence of distant metastases can be accepted as positive proof of this.

Gorbman (1947) fed mice of 2 strains, A and C57, on a diet containing 0·1 per cent thiouracil. A vast series of changes were noted in the thyroids of these animals, which were killed at periods varying from 7 to 566 days. Of the 22 survivors killed after 500 days, he found pulmonary deposits resembling thyroid tissue in 7, all strain A mice. These little nodules were not accepted as true metastases with neoplastic potentiality, but rather as fragments of hyperplastic thyroid tissue that had acquired an intravascular position in a very active gland, and had been then swept into the venous circulation.

Dalton, Morris and Dubnik (1948) fed 24 strain C3H mice on a diet containing 0·5 per cent thiouracil. After 362 days pulmonary deposits were found in 10 of them. Once again the intravascular position of these foci and their endothelial investment was noted. Repeating the experiment with 32 strain C mice only one instance of pulmonary involvement was encountered, though in these animals nodular hyperplasia of the thyroid was more conspicuous than in strain C3H mice (Dalton, Morris, Striebich and Dubnik, 1950). It was essential to assess the neoplastic nature of this thyroid tissue more conclusively, so Morris and Green (1951) transplanted it into young mice on a diet containing thiouracil. After 3 to 6 months, when the tissue had increased sufficiently in size, it was retransplanted into other mice on a similar diet. After several transplantations there appeared lines of thyroid tissue capable of growth in mice fed on a normal diet. Ultimately some of these tumours metastasised to the lungs and killed the animals. It could now be concluded that autonomy has been attained, in that growth could occur without any additional requirement of thyrotrophic hormone.

In the present experiment a correlation between the degree of induced thyroxin deficiency and the gamut of hyperplastic response has been made. The administration of methylthiouracil alone produced profound hyperplasia of a characteristically papillary type. When it was combined with a low iodine diet, focal papillary adenomata were encountered in almost half the thyroids examined. The gradual transformation from diffuse acinar hyperplasia with papillary ingrowths to

fully established papillary hyperplasia is followed in turn by the appearance of nodules of extreme hyperplasia. It is in these areas that focal adenomata arise.

The difficulty in inducing malignant change in such glands is again emphasised, though in one case there was clear-cut and decisive cancerous transformation. Had the investigation been terminated later, it is quite possible that malignancy might have been encountered more frequently. To this purpose another group of mice is being treated similarly at present and in these the extreme thyroxin deficiency will be allowed to act for a greater period of time.

SUMMARY

One hundred C57 mice were divided into 3 groups :

(*a*) A control group of 25 mice fed on a stock diet and tap water.

(*b*) A group of 50 mice fed on a stock diet and distilled drinking water containing 0·05 per cent methylthiouracil.

(*c*) A group of 25 mice fed on a low iodine diet and distilled drinking water containing 0·05 per cent methylthiouracil.

After 480 days no significant changes were found in the thyroid glands of the control group.

In the second group extreme hyperplasia of a typically papillary type was encountered, but there was no evidence of neoplasia.

In the third group focal papillary adenomata were found in 11 of the 25 mice, but in none was there any tendency to local infiltration or distant metastasis. One mouse, however, did succumb to an adenocarcinoma of the thyroid that metastasised to the lungs.

The extreme difficulty in producing thyroid neoplasms in mice even under the most intense stimulation is demonstrated once again.

We are grateful to Professor G. J. Cunningham for his interest and assistance ; to Miss June Hunter, of the Medical School, University of Otago, New Zealand, for help in planning the experiment ; to Dr. J. Craigie, of the Imperial Cancer

EXPLANATION OF PLATES

FIG. 1.—Normal thyroid gland in one of the control group. H. and E. ×65.

FIG. 2.—Pronounced acinar hyperplasia with papillary invaginations into some acini. H. and E. ×65.

FIG. 3.—A later stage in the evolution of papillary hyperplasia from acinar hyperplasia. H. and E. ×65.

FIG. 4.—The pattern of fully-developed papillary hyperplasia. H. and E. ×65.

FIG. 5.—A circumscribed nodule of papillary hyperplasia resembling an adenoma. The cellular pattern is quite uniform. H. and E. ×65.

FIG. 6.—The regular cellular arrangement in papillary hyperplasia. H. and E. ×200.

FIG. 7.—More pronounced hyperplastic pattern with some large cells having giant nuclei. H. and E. ×200.

FIG. 8.—Solid cell hyperplasia. H. and E. ×70.

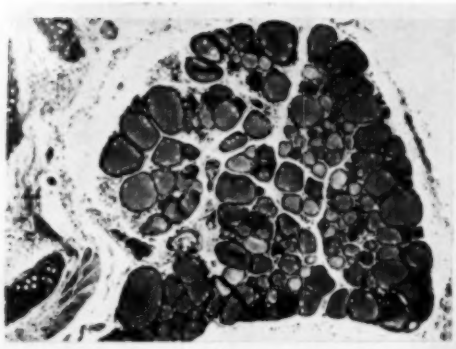
FIG. 9.—A circumscribed papillary adenoma. The arrangement and morphology of the cells is quite different from that of the surrounding tissue. H. and E. ×70.

FIG. 10.—The distinctive cellular structure of a papillary adenoma. H. and E. ×200.

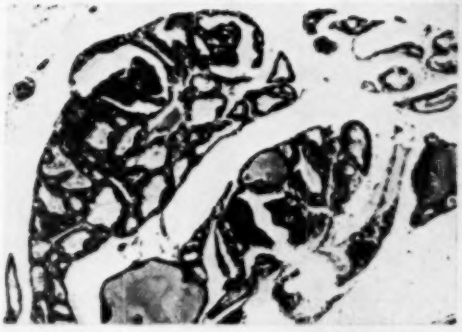
FIG. 11.—A particularly exuberant papillary adenoma. H. and E. ×70.

FIG. 12.—A rather poorly differentiated adenocarcinoma showing a papillary pattern in some areas and an acinar one elsewhere. H. and E. ×70.

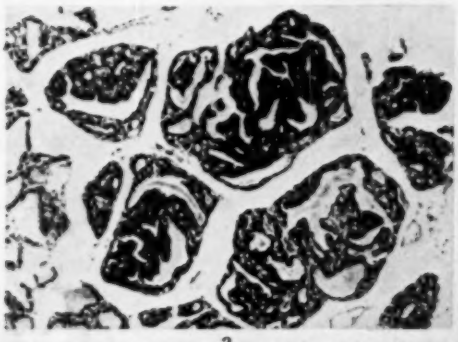
FIG. 13.—A pulmonary metastasis. It has a much better preserved acinar pattern than the primary tumour. H. and E. ×70.



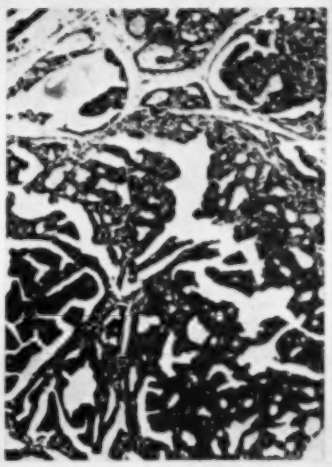
1



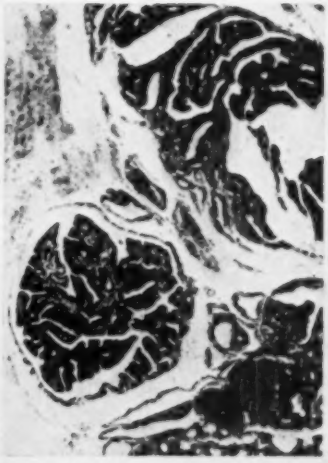
2



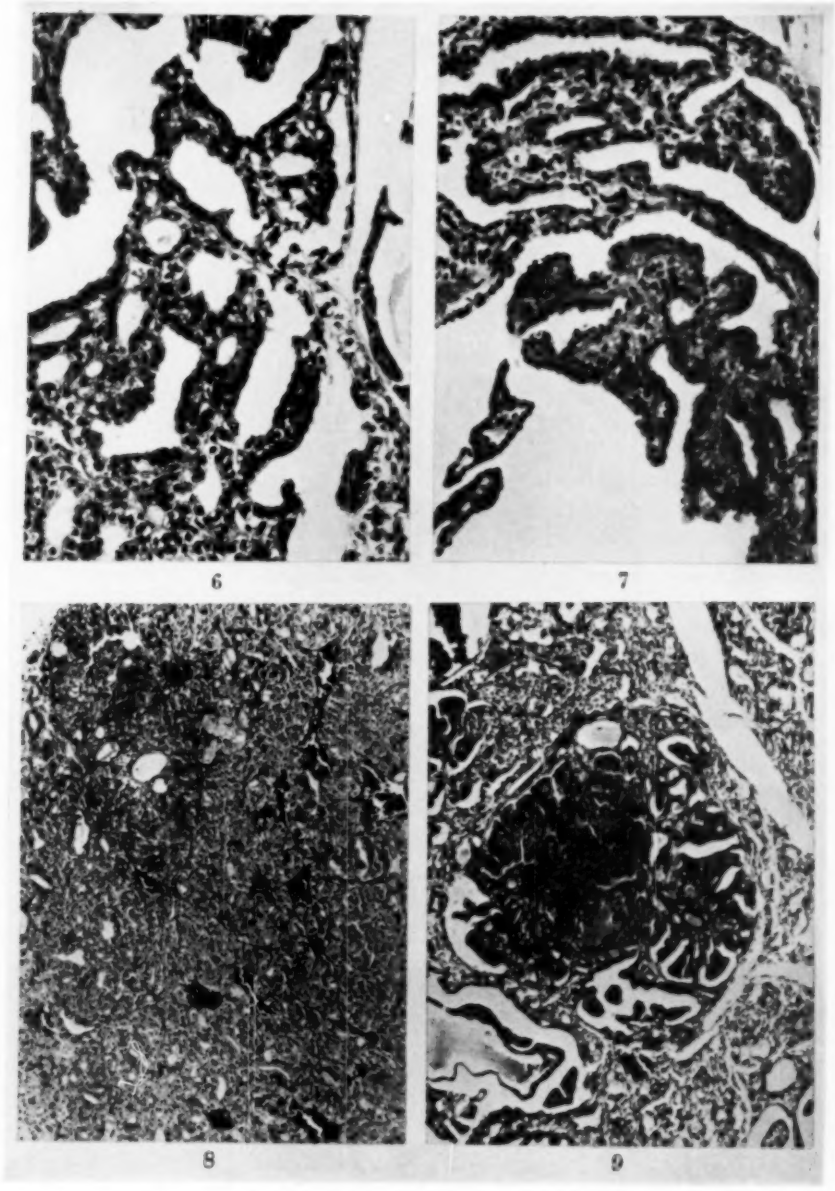
3



4

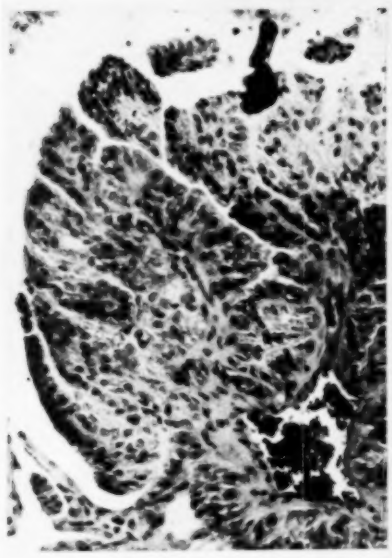


5

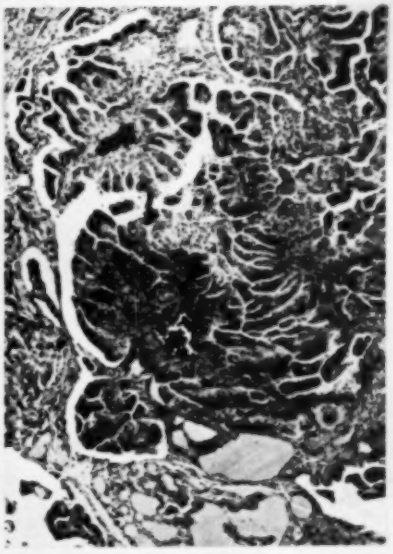


6 7

8 9

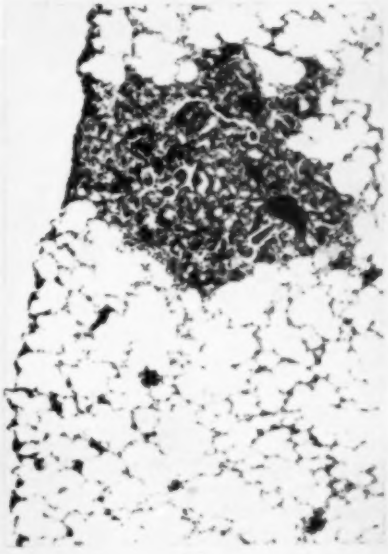


10



11

12



13

Research Fund, for advice about the low iodine diet ; and to Mr. A. L. E. Barron for the photomicrographs.

REFERENCES

Astwood, E. B., Sullivan, J., Bissell, A. and Tyslowitz, R.—(1943) *Endocrinology*, **32**, 210.
Axelrad, A. A. and Leblond, C. P.—(1952) *Canad. med. Ass. J.*, **67**, 675.—(1955) *Cancer*, **8**, 339.
Bielschowsky, F.—(1955) *Brit. J. Cancer*, **9**, 80.
Burt, A. S., Landing, B. H. and Sommers, S. C.—(1954) *Cancer Res.*, **14**, 497.
Dalton, A. J., Morris, H. P. and Dubnik, C. S.—(1948) *J. nat. Cancer Inst.*, **9**, 201.
Idem, Morris, H. P., Striebich, M. J. and Dubnik, C. S.—(1950) *Ibid.*, **11**, 391.
Gorbman, A.—(1947) *Cancer Res.*, **7**, 746.
Hall, W. H. and Bielschowsky, F.—(1949) *Brit. J. Cancer*, **3**, 534.
Morris, H. P. and Green, C. D.—(1951) *Science*, **114**, 44.
Remington, R. E.—(1937) *J. Nutr.*, **13**, 223.
Russfield, A. B.—(1955) *J. clin. Endocrin.*, **15**, 1393.

CARCINOGENESIS IN THE SKIN OF THE HEDGEHOG

F. N. GHADIALLY

From the Department of Pathology, The University, Sheffield

Received for publication March 31, 1960

It has been shown (Whiteley, 1957; Ghadially, 1958, 1959) that many carcinogen-induced cutaneous tumours of laboratory rodents arise from pilo-sebaceous follicles and are morphologically similar to the kerato-acanthomas of man. Pilo-sebaceous follicles show considerable variations of morphology and distribution in various animal species. The spines of the hedgehog are homologous to the hairs of laboratory rodents and arise from very large follicles set well apart from one another, while rodent skin is thickly populated by comparatively small hair follicles. It was felt that the study of carcinogenesis in a skin so different from that commonly employed might yield valuable information regarding the histogenesis of carcinogen-induced cutaneous tumours. Hitherto the hedgehog has not been employed for the study of chemical carcinogenesis.

METHODS

The spines on the dorsum of six hedgehogs were removed with the aid of nail clippers, and an area of skin about 2 square inches was painted with a solution of 2 per cent w/w 9 : 10 dimethyl-1 : 2 benzanthracene (DMBA) in equal parts of lanoline and paraffin. A total of 30 applications of carcinogen was administered over a period of eleven months, and the animals were observed for a further 2-month period. Four animals died during the early stages of the experiment (3 to 6 months). Of the two that survived one developed tumours during the eleventh month; the other died about a month later without producing any tumours. The animal with the tumours was killed approximately 13 months after commencement of the experiment. The tumours and pieces of adjacent skin were removed and sections stained with haematoxylin and eosin were prepared in the usual manner.

RESULTS

A number of tumours were produced on the skin of the hedgehog at the site of painting (Fig. 1). One outgrowing papilloma (Fig. 2) was found arising from the epidermis. Typical type I kerato-acanthomas were not found but three small horns similar to that shown in Fig. 3 were discovered. Since such superficially placed horns with a cup-shaped base have been found to evolve from type I kerato-acanthomas in other species (Ghadially, 1958, 1959), it is possible that we are here witnessing the same phenomenon. The base of one of the large horns seen in Fig. 1 was carcinomatous and had produced three small distinct foci of secondary growth in a lymph node. In two of these foci the tumour cells resembled basal cells (Fig. 4) while in the other squamous differentiation and keratin form-

ation was evident (Fig. 5). Numerous melanin-containing phagocytes were present in the lymph node but no melanocytes were detected.

The majority of the lesions seen in this hedgehog were type III kerato-acanthomas (Ghadially, 1958, 1959). Many stages of evolution of this lesion were detected. The mature deeply placed berry-shaped lesion is shown in Fig. 6. Regressing lesions with marked fibrosis and disintegrating epithelial columns of pseudo-carcinomatous appearance, strikingly similar to those seen in kerato-acanthomas of man and other experimental animals were also found. It has been claimed that the type III kerato-acanthoma probably arises from the hair germ, or from the deeper part of the hair follicle which is developed afresh at each hair growth cycle from the hair germ (Whiteley, 1957 ; Ghadially, 1958). Hitherto, morphological evidence supporting such a concept has been lacking primarily because a small early lesion starting at the base of the follicle cannot be detected early enough macroscopically in the skin of the experimental animals. Fig. 7 and 8 show a deeply placed cystic epithelial lesion which was discovered accidentally. It is almost certainly an early stage development of a type III kerato-acanthoma. Whiteley (1957) found that in the rabbit type III kerato-acanthomas developed during the resting phase (telogen) of the hair growth cycle. The same seems to be true in the case of this hedgehog for the spine follicles in the surrounding skin were in telogen.

DISCUSSION

The significant fact that emerges from this study of hedgehog tumours is that in spite of such marked obvious morphological differences between the skin of this animal and that of laboratory rodents and man, the types of benign epithelial tumours produced by carcinogenic action are essentially similar in all these species. It has sometimes been argued that the results of carcinogenic studies in rodent skin thickly covered by fur can hardly be applicable to the skin of man. Whiteley (1957) and Ghadially (1958, 1959) have already demonstrated the astonishing similarly of both behaviour and morphology of the kerato-acanthomas of man and experimentally produced tumours in rabbit, mouse and hamster skin and now we observe that the same is also true in the case of an insectivore—the hedgehog. Kerato-acanthomas have also been produced on the feather-bearing skin of birds (Rigdon, 1959). The fact that morphologically similar tumours occur in such a vast variety of species with different types of hair follicles and their homologues does not detract from the argument that kerato-acanthomas arise from these structures, for it has been shown that when the glabrous skin of the duck's foot (Rigdon, 1956) or the exteriorised cheek pouch epithelium of the hamster (Ghadially and Illman ,1960) is painted with carcinogen many papillomas but no kerato-acanthomas are produced.

An interesting incidental finding was the early stage of development of a type III kerato-acanthoma (Fig. 7, 8). Experimental evidence derived from the study of the hair growth cycle and carcinogenesis (Whiteley, 1957) had already indicated that a deeply placed berry-shaped lesion probably arising from the hair germ occurs in the skin of rabbits painted with carcinogens, and a morphological analysis of these tumours (type III kerato-acanthomas) in man and laboratory rodents led Ghadially (1958, 1959) to essentially the same conclusions. The importance of the tumour illustrated in Fig. 7 lies in the fact that here we have a lesion which has almost certainly arisen in this fashion. We can rule out the possibility

that this tumour has arisen from the epidermis or from the superficial part of the spine follicle, for serial sections have demonstrated no point of contact between these structures and the tumour. The position which this tumour occupies, under the quiescent spine follicle and the erector spinal muscle is consistent with the position where one would expect a tumour from the spine germ to lie. (The hair germ, or the spine germ and the deeper part of the follicle which is developed from this structure lie below the plane of attachment of the erector muscle of the follicle.) This lesion was found in an animal in which many type III kerato-acanthomas developed. It is therefore not surprising that an early lesion was also found in this animal. However since this tumour was found in an animal which also had a carcinoma it can be argued that we are here witnessing an infiltrative or metastatic extension of this tumour. This seems unlikely for the following reasons. (*a*) Serial sections show no connection between this tumour and the carcinoma. (*b*) The lesions produced by metastatic squamous carcinoma are usually irregular and infiltrative and not rounded and sharply demarcated as the one shown in Fig. 7. (*c*) This tumour resembles a kerato-acanthoma in every detail, for this is a cystic lesion with a central core of keratin surrounded by irregular squamous epithelium showing pseudo infiltration. The characteristic lymphocytic infiltration is also seen at the periphery of this tumour.

SUMMARY

It has been shown that tumours can be produced in the skin of the hedgehog, which is an insectivore, by repeated applications of DMBA.

Many kerato-acanthomas, a papilloma, and a carcinoma with secondary deposits in a lymph node were produced.

In spite of the marked morphological differences that exist between the skin of the hedgehog and the skin of man and laboratory rodents the tumours that arise show a marked morphological similarity.

An early stage of development of a type III kerato-acanthoma was found and studied by serial sectioning. The appearances seen support the concept of Whiteley and Ghadially that these tumours arise from the hair germ, or the deeper part of the hair follicle.

EXPLANATION OF PLATES

FIG. 1.—Tumours produced on the dorsal skin of a hedgehog by 9 : 10 dimethyl 1 : 2 benzanthracene. × ¾.

FIG. 2.—Papilloma. ×24.

FIG. 3.—Cutaneous horn. ×10.

FIG. 4.—Secondary deposit in lymph node showing basal-cell differentiation. ×220.

FIG. 5.—Secondary deposit in lymph node showing squamous-cell differentiation. ×255.

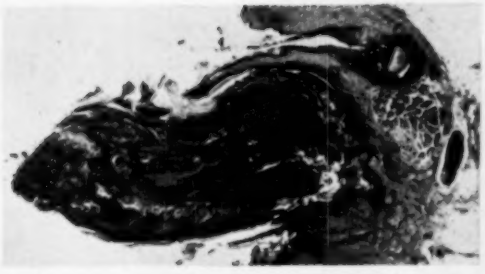
FIG. 6.—A portion of a mature type III kerato-acanthoma. ×7.

FIG. 7.—Early stage of development of a type III kerato-acanthoma. This is a cystic tumour with a central mass of keratin. The tumour is seen to lie below a keratin plugged spine follicle and the erector spinae muscle. ×14.

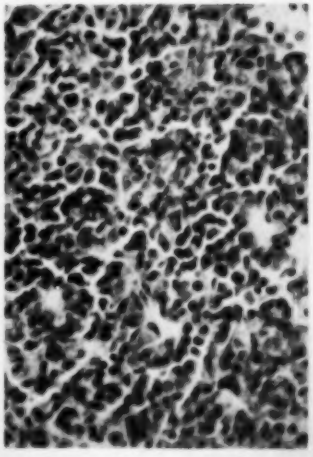
FIG. 8.—High power from cystic wall of tumour shown in Fig. 6. The central keratin-filled cavity lies to the bottom of the picture. From bottom to top one can see keratin masses, neoplastic squamous cells, and lymphocytic infiltration at the periphery of the tumour. ×135.

1

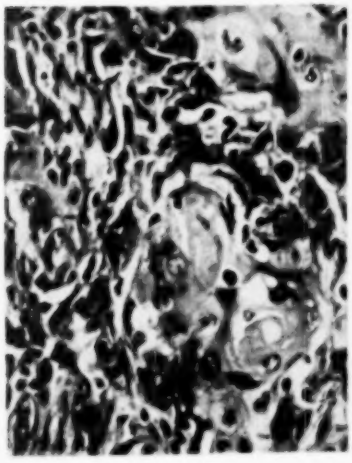
2



3

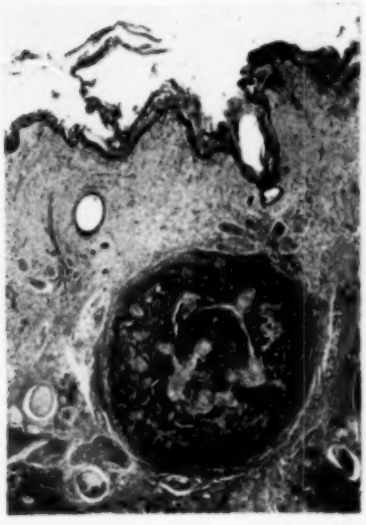


4



5

6



7

8

Ghadially.

I am indebted to Professor D. H. Collins for helpful advice and criticism and to Mr. J. H. Kugler, T. L. Platts, A Whitaker, J. N. Carver and S. Wall for technical assistance. The work was supported by grants from the University of Sheffield Medical Research Fund and from the British Empire Cancer Campaign.

REFERENCES

GHADIALLY, F. N.—(1958) *J. Path. Bact.*, **75**, 441.—(1959) *Ibid.*, **77**, 277.
Idem AND ILLMAN, O.—(1960) *Ibid.* In press.
RIGDON, R. H.—(1956) *Cancer Res.*, **16**, 804.—(1959) *Arch Derm. Syph., N.Y.*, **79**, 139.
WHITELEY, H. J.—(1957) *Brit. J. Cancer*, **11**, 196.

FLUORESCEIN-GLOBULIN AFFINITIES OF THE SHOPE PAPILLOMA

C. J. LOUIS

From the Department of Pathology, University of Melbourne, Melbourne, Australia

Received for publication April 30, 1960

THE fluorescein-globulin method of staining which distinguishes neoplastic from non-neoplastic cells has now been employed in a sufficiently large number of cases to demonstrate that it applies consistently both to chemically induced tumours in animals and to naturally occurring tumours in both animals and man (Louis, 1957*b*, 1958*a*, 1958*b* ; Louis and Varasdi, 1960). The test also affords a means of differentiating acute leukaemia from the chronic form (Louis, 1957*c*, 1958*d*).

It has been shown that all normal tissue cells of vertebrates and some invertebrates stain well and fluoresce brightly provided they contain an adequate amount of cytoplasm. The few exceptions that do not stain, namely : resting connective tissue cells, red blood cells, cells of the central nervous system and cells in tissue culture, have been discussed previously (Louis, 1958*c* ; Louis and White, 1960). The significance of the staining reaction although not yet completely understood has been attributed to protein-protein interactions between the basic cytoplasmic protein(s) of the normal tissue cells and the labelled serum proteins which have been rendered less basic by the conjugation process (Creech and Jones, 1941*a*, 1941*b* ; Hopkins and Wormall, 1933). That no serological implication is concerned in this reaction has been demonstrated by the observation that identical results could be obtained by using normal rabbit globulin (Hughes, Louis, Dineen and Spector, 1957), albumin and globulin fractions of many different species and even of the same animal (King, Hughes and Louis, 1958, 1959) and finally by egg albumen (Hughes and Louis, 1959). All malignant cells, on the other hand, even though containing large volumes of cytoplasm, failed to stain. Concomitant with the onset of the malignant change tumour cytoplasmic proteins become less basic (Eldredge and Luck, 1952 ; de Lamirande, Allard and Cantero, 1953 ; Sorof and Cohen, 1951). Hence it is to be expected that these proteins would have a decreased capacity to form complexes with the fluorescein-globulin conjugates. This has been found to be the case in a large number of tumours examined. In addition, certain presumably " premalignant " but morphologically normal cells were found not to stain.

Because these observations relate only to chemically induced and naturally occurring tumours it was decided to investigate a virus induced tumour, partly to study virus infected cells and partly to see if this type of neoplastic change differs from the chemically induced variety. Thus the results of the staining affinities of the Shope papilloma during its course of development are presented and discussed.

METHODS AND MATERIALS

Papilloma virus

The virus for inoculation was supplied by Dr. Richard E. Shope of the Rockefeller Institute in tissue slices of freshly removed warts stored in 50 per cent. glycerol. The tumour slices were ground with washed sand and normal saline to yield a 10 per cent suspension which was subsequently filtered through filter paper (Whatman No. 1). This filtrate was used for inoculation.

Inoculation of rabbits

Twenty-four young white rabbits of mixed breed had small areas shaved on the dorsum of the distal halves of both ears followed by light scarification with sand paper. Inoculation was effected in some by rubbing the virus suspension into these areas, whilst in others by intradermal injection of 0·1 ml.

A second group of 12 rabbits had similar areas of skin prepared which were painted daily 5 days per week for approximately 12–14 weeks with the following carcinogens: 3 : 4-benzpyrene, 1 : 2 : 5 : 6-dibenzanthracene and 20-methylcholanthrene. These carcinogens were made up in 0·2 per cent solution with benzene. When small wart-like projections appeared the carcinogen application was stopped and each rabbit was given 2·0 ml. of the infected filtrate intravenously.

Production of hepatomata

Adult Sprague Dawley rats weighing 150–200 g. were used. Hepatomata were induced by feeding 0·06 per cent 4-dimethylaminoazobenzene in a 20 per cent casein diet containing 2 mg. of riboflavine per kg. Rats were killed under ether anaesthesia at 2 weeks and then at weekly intervals until 12-15 weeks when liver tumours appeared. These have been described in detail elsewhere (Hughes, 1958; Hughes, Louis, Dineen and Spector, 1957) and are used here for comparison with the rabbit tumours (Table I).

Preparation of sera

The globulin fraction was extracted from the serum of both inoculated and non-inoculated rabbits by precipitation with half saturated ammonium sulphate and after dialysis was conjugated with fluorescein isocyanate (Isomer I) by the method of Coons and Kaplan (1950).

Preparation of tissue sections

In most cases small pieces of biopsy material were taken from the margins of the tumours and immediately snap frozen by dropping in isopentane precooled to −75° C. in an ethanol-dry ice mixture. From these unfixed frozen sections were cut at 5–7 μ by a method previously described (Louis, 1957*a*).

Fluorescence staining, microscopy and photography

Before staining, the free fluorescein derivatives were removed from the conjugated sera with ethyl acetate (Dineen and Ada, 1957). The dried tissue sections were treated with the labelled globulins for 10 minutes at room temperature, washed gently in three changes of buffered saline, pH 7·3, and examined in ultra-

violet and blue light with a Leitz fluorescence microscope. Appropriate areas were selected and photographed using ultra-violet light, then the same sections were fixed in 10 per cent formol-saline, stained with haematoxylin and eosin and, for comparison, the same areas were rephotographed using visible light.

RESULTS

The macroscopic and microscopic observations on papillomatosis experimentally induced in this series of animals were similar in all cases and good accounts have been given by Shope (1933), Rous and Beard (1935) and Syverton (1952). For the purpose of this study the development of these cutaneous tumours is treated in four stages :

(*a*) The phase of active epidermal proliferation ;
(*b*) the quiescent phase ;
(*c*) a premalignant phase ; and
(*d*) the malignant phase.

Unfixed frozen sections prepared from all tissues investigated were examined first in the unstained state to exclude any intrinsic fluorescence present independently of the use of the stain. As with previous experiments it was observed that elastic tissue and the ground substance of cartilage emit a bright yellow autofluorescence and keratin pale blue. A particular effort was made to avoid confusing these with the characteristic green fluorescence which results after staining with the fluorescein-labelled sera.

Normal rabbit epidermis

All the cellular layers of the stratified squamous epithelium in rabbit skin showed a strong affinity for fluorescein-globulin complexes and emitted a bright green fluorescence in ultra-violet light (Fig. 1 and 2). There was a uniform distribution of the dye within the cells in which the cytoplasm, but not the nucleus, fluoresced. The cells lining the hair follicles and sudoriferous glands also stained uniformly.

Proliferating epidermis

This stage of development became apparent macroscopically between the 10th and 14th day after inoculation as small papular vesicles which steadily progressed and developed into large verrucous masses (Fig. 3). This phase lasted approximately 5 months. Microscopically, in their well developed stage, the warts showed considerable thickening of the epidermis with papillary outgrowths from the surface and gross keratin formation (Fig. 4). There was active proliferation of the prickle cell layer with increase in the number of cells and numerous mitotic figures. Many of these cells contained pigment. These cells, however, were regular in form and the deeper layers clearly demarcated from the underlying connective tissue stroma. Small collections of wandering cells, principally of the small round cell type, were present in the superficial dermis.

Histochemically, after staining with the conjugated dye, the hyperplastic cells and those which were undergoing mitosis fluoresced brightly and uniformly as normal epithelium (Fig. 5 and 6).

Premalignant phase

About 6 months after inoculation the growths stopped growing macroscopically which was evidenced microscopically by the gradual decrease in the number of cells, absence of mitotic figures and desquamation of the superficial epidermal layers. This persisted for a further 6 months, during which period some involution took place (Fig. 7 and 8).

The fluorescence staining characteristics in the beginning of this phase were similar to those observed with normal and hyperplastic epithelium—namely, a uniform staining of the epithelial cells. At the 10th month there were observed groups of epithelial cells which showed a diminished affinity for the stain and failed to fluoresce in ultra-violet light (Fig. 9 and 10). These islands varied in size and contained anything from 6 up to 40 cells. No morphological evidence of malignancy was detected when these sections were stained with a routine stain such as haematoxylin and eosin. Again the cells were regular in form throughout and the basal layers sharply demarcated from the underlying stroma.

Malignant phase

Irregular " islands of loss " of staining were observed until 15 months without histological evidence of any malignant development. At about this time evidence of invasion became apparent. Masses of cells, arranged in small groups and strands and showing epidermoid differentiation, extended into the underlying stroma. These were irregular in shape, size and nuclear densities and showed many mitotic figures. Initially these were confined to the superficial dermis (Fig. 11–14). Later collections of cells were seen invading the cartilaginous plate (Fig. 15–18) and finally penetrated through the ventral aspect of the rabbits' ears. Here a clear-cut difference was observed between the non-fluorescing invading tumour tissue and the brightly fluorescing invaded ventral epidermis (Fig. 19 and 20).

Exposure to chemical carcinogens

In rabbit ears pretreated with carcinogens similar microscopic and macroscopic changes were observed but the malignant transformation after intravenous injection of the virus occurred much earlier than in the first group. The epidermal cells showed strong affinities for both conjugated dyes up to the 6th month stage of development when islands of loss of staining could be demonstrated. These islands persisted only for 3 months before obvious malignant changes became apparent.

The resultant tumours appeared more active than the previous ones in that invasion was much more rapid and metastases more widespread.

In sections taken from both primary and secondary nodules no fluorescence was emitted by the tumour cell cytoplasm.

Rat liver after 4-dimethylaminoazobenzene administration

Examination of sections from the livers of rats fed 4-dimethylaminoazobenzene showed that all the parenchymal cells stained uniformly up to the 3rd week of carcinogen administration. After this irregular islands of morphologically normal parenchymal liver cells were observed not to stain. These islands persisted for 12–15 weeks when hepatomata developed. All the tumour cells in these growths

failed to stain with the labelled sera in contrast to the brightly fluorescing adjacent normal liver cells.

DISCUSSION

Although numerous studies on the nature of neoplasia have been made and much information collected, an understanding of the fundamental processes involved is still distant. During the last century numerous vague aetiological factors were held responsible for the neoplastic change but these have recently become replaced by a number of distinct agents, which have been subdivided into chemical, physical (β-, γ-, and related rays) and organismal (particularly the viruses).

Much of our present knowledge of carcinogenesis has stemmed from the discovery and study of the chemical carcinogens. To date many such substances have been discovered but it has been shown that often a carcinogen will affect only a particular organ of a particular species. This effect may be produced by local application, oral administration or by injection parentally. Repeated observations with three groups of substances, namely: aminoazo dyes (Miller and Miller, 1947), aromatic polycyclic hydrocarbons (Heidelberger and Moldenhauer, 1957; Heidelberger and Weiss, 1951) and aminofluorenes (Miller and Miller, 1952), have shown that the carcinogen is taken up by the cells of the susceptible tissue. Differential high speed centrifugation of the tissue homogenates and subsequent electrophoresis (Eldredge and Luck, 1952; de Lamirande, Allard and Cantero, 1953; Sorof and Cohen, 1951) indicated that the carcinogen became firmly bound to a protein or protein complex present in the soluble fraction of the cell cytoplasm. Investigation of the tumours which developed subsequently showed that the tumour cells not only lacked this dye but also the protein(s) which was present in the parent cell and to which the carcinogen presumably became attached (Miller and Miller, 1947). Thus there was a clearly defined and easily reproducible difference between a normal and a malignant cell. This difference has been demonstrated histochemically in a variety of naturally occurring and chemically induced tumours using fluorescein-globulin conjugates (Louis, 1957*b*; Louis, 1958*a*; Louis, 1958*b*; Louis and Varasdi, 1960). The normal cells, which contained the basic protein(s), reacted with the labelled dye and emitted a bright green fluorescence in ultra-violet light whereas the malignant counterparts did not. In most sections prepared from the livers of rats fed 4-dimethylaminoazobenzene non-staining islands of cells were seen. The failure of these islands of morphologically normal but perhaps premalignant parenchymal cells to fluoresce appeared to be of great aetiological significance.

Our knowledge of virus carcinogenesis, on the other hand, is relatively meagre. Difficulties encountered in extraction and purification of these viruses are necessarily responsible for the poor understanding of this group of neoplasms. Since the Shope papilloma virus was readily available and provided a good example of the relationship of viral agents to benign and malignant growths it has been studied in detail in the present paper. This virus, like the chemical carcinogens, has been shown to possess strong cytotropism causing only epidermal cells of rabbits to proliferate and, after a latent period, to become malignant (Shope, 1933). In the present investigation examination of many examples of epidermal hyperplasia which had progressed to the twelve month stage of development showed strong affinities for the conjugated dyes and fluoresced as brightly as the adjacent normal

epidermis. This was a normal reaction and conformed with those made on naturally occurring hyperplastic conditions in man, on the early stages of chemically induced skin tumours in mice (Louis, 1958*b*) and on the hyperplastic tissue of the regenerating rat liver (Louis, 1957*d*). All forms of obviously malignant tissue in this series failed to fluoresce. This was particularly striking in the early stages where invasion was confined to the superficial dermis. Here groups of cells showing good squamoid differentiation with well-developed keratin nests and intercellular prickles lacked all affinities for the fluorescent stain and failed to show a positive staining reaction (Fig. 11–18). Here also islands of morphologically normal epidermal cells have been observed which have shown the characteristic loss of staining (Fig. 7–10). The persistence of these islands, their possession of fluorescence staining characteristics similar to those of frank epidermoid carcinoma cells and the subsequent development of carcinoma at these sites suggests strongly that they are premalignant foci.

In all tumours, both experimentally induced and naturally occurring, examined by this method of study, the difference from normal tissue has been clear-cut and well defined. Since this difference appears to be due to the absence from the malignant cell of a protein complex, it seems probable that such a change would not necessarily be an abrupt one. Indeed, the present and previous investigations indicate that the final form of tumours is due to a series of changes. The nature of such changes occurring in the cells during the preneoplastic phase and concomitant with the development of malignancy is still unknown. The first histochemical evidence that a change has occurred is indicated by the appearance of persistent islands of morphologically normal cells with a diminished affinity for fluorescein-globulin complexes, a feature common to both chemically and viral induced tumours. Thus if the changes occurring during viral carcinogenesis are compared with those induced with a chemical carcinogen such as the aminoazo dyes (Table I) the changes leading to malignancy are found to be similar. Furthermore, the Shope virus can act synergistically with certain carcinogenic hydrocarbons (Rogers and Rous, 1951). In the present investigation, this phenomenon has been demonstrated with 20-methylcholanthrene, 3 : 4-benzpyrene and 1 : 2 : 5 : 6-dibenzanthracene. Intravenous injection of papilloma virus induces malignant tumours in areas of skin which have previously been treated with

TABLE I.—*Comparison of Fluorescence Staining of Shope Rabbit Tumours and Hepatomata Induced in Rats by 4-Dimethylaminoazobenzene*

	Shope virus	4-DAB
Species specificity	Rabbit	Rat.
Organ specificity	Epidermis	Liver.
Presence of carcinogen in normal cells	Can be extracted from tumours up to 9 months after inoculation	Firmly bound to soluble cytoplasmic proteins for 9–12 weeks. Tissue turns pink on acidification.
"Islands of loss of fluorescence staining" of morphologically innocent cells	Detected between 12th–15th month after inoculation and before development of frank malignancy	Detected 4–12 weeks after feeding 4-DAB.
Presence of carcinogen in tumour cells	Malignant change occurs at approximately 18 months. No virus extracted from tumour tissue. Tumour cells fail to fluoresce	Hepatomas appear at approximately 12 weeks. Tumour tissue does not turn pink on acidification. Tumour cells do not fluoresce.

polycyclic hydrocarbons. In these cases the tumours appear sooner, they are larger and metastasize more readily. Although tumours induced in this manner are similar to those induced with simple virus inoculations, Smith, Kid and Rous (1952) were not able to extract an infective filtrate from these. However, in spite of such similarities there may be a distinct difference between the presumed loss of a dye binding cytoplasmic protein and the situation in which there is failure to extract an exogenous virus-like agent from a neoplasm.

In résumé, therefore, it may be said that the fluorescein-globulin affinities of normal, hyperplastic and neoplastic cells of a virus induced papilloma are identical to those of chemically induced and naturally occurring neoplasms. The distinction

EXPLANATION OF PLATES

FIG. 1.—Rabbit skin. Unfixed frozen section stained with fluorescein-globulin complex and showing bright uniform fluorescence of cytoplasm of epithelial cells; vacuoles and nuclei do not stain. ×120.

FIG. 2.—Same section and area as shown in Fig. 1 after fixation in 10 per cent formol-saline and stained with haematoxylin and eosin for comparison. ×120.

FIG. 3.—Rabbit ear showing young papilloma (3 months after inoculation). ×3.

FIG. 4.—Paraffin section from tumour in Fig. 3 showing the typical structure of benign papilloma. Haematoxylin and eosin. ×30.

FIG. 5.—Unfixed frozen section prepared from tumour in Fig. 3 and stained with fluorescein-globulin complex. The cytoplasm of epithelial cells stains uniformly. ×220.

FIG. 6.—Same section and area as shown in Fig. 5 subsequently fixed in formol-saline and stained with haematoxylin and eosin for comparison. ×220.

FIG. 7.—Shope papilloma 10 months after inoculation. ×1·5.

FIG. 8.—Paraffin section from tumour in Fig. 7 showing hyperplastic epithelium which is regular in form throughout and basal layers clearly demarcated from dermis. Haematoxylin and eosin. ×30.

FIG. 9.—Unfixed frozen section prepared from margin of tumour in Fig. 7 and stained with fluorescein-globulin complex. Note some cells fluoresce brightly, some show diminished fluorescence and some complete absence of fluorescence ("islands of loss"). ×240.

FIG. 10.—Same section and area as shown in Fig. 9 subsequently fixed in 10 per cent formol-saline and stained with haematoxylin and eosin for comparison. Note that the non-fluorescing cells are morphologically innocent. ×240.

FIG. 11.—Shope papilloma 15 months after inoculation showing breakdown of surface keratin and early ulceration. ×1·5.

FIG. 12.—Paraffin section from tumour in Fig. 11. There is early invasion of dermis by well differentiated squamous epithelium. The cartilaginous plate is intact. ×15.

FIG. 13.—Unfixed frozen section prepared from margin of tumour in Fig. 11 and stained with fluorescein-globulin complex showing autofluorescence (yellow) of ground substance of cartilaginous plate. Note collection of cells outlined in non-fluorescing background. ×240.

FIG. 14.—Same section and area as shown in Fig. 13 subsequently fixed in 10 per cent formol-saline and stained with haematoxylin and eosin for comparison. The non-fluorescing cells, although irregular in form, show good squamoid differentiation. ×240.

FIG. 15.—Shope papilloma 18 months after inoculation showing extensive ulceration. ×1.

FIG. 16.—Paraffin section from tumour in Fig. 15. Groups of cells are seen invading through to cartilaginous plate. ×30.

FIG. 17.—The rectangular area in Fig. 16. Fluorescence photomicrograph showing bright autofluorescence (yellow) of ground substance of cartilage and outlines of non-fluorescing cells in the background. ×240.

FIG. 18.—Same section and area as in Fig. 17 subsequently fixed in formol-saline and stained with haematoxylin and eosin for comparison. The collections of non-fluorescing cells are the characteristic neoplastic tissue. ×240.

FIG. 19.—Margin of Shope papilloma 2 years after inoculation. Fluorescence photomicrograph showing bright fluorescence of two digitations and outlines of non-fluorescing cells. ×240.

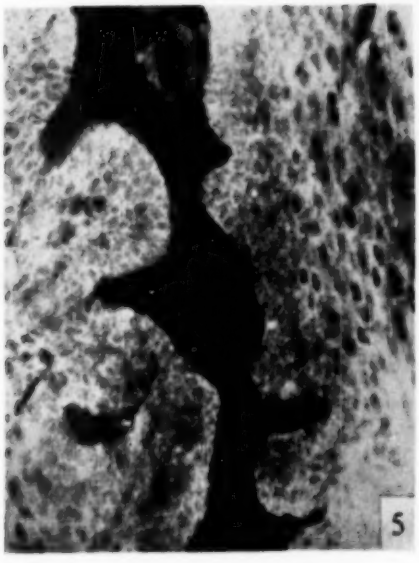
FIG. 20.—Same section and area as in Fig. 19 subsequently fixed in formol-saline and stained with haematoxylin and eosin. The fluorescing areas represent the normal epidermis whereas the non-fluorescing area constitutes the typical tumour tissue. ×240.

1

2

3

4


5

6

Louis.

7

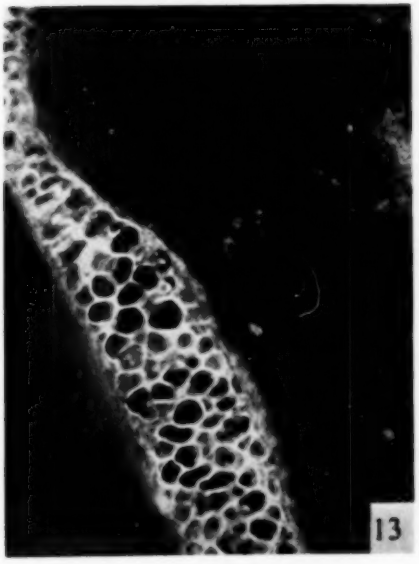
8

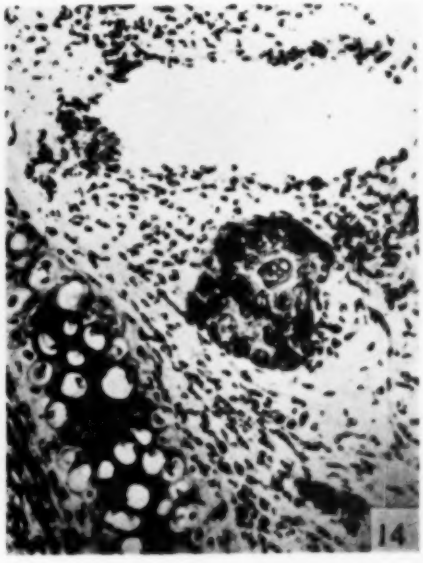
9

10

11

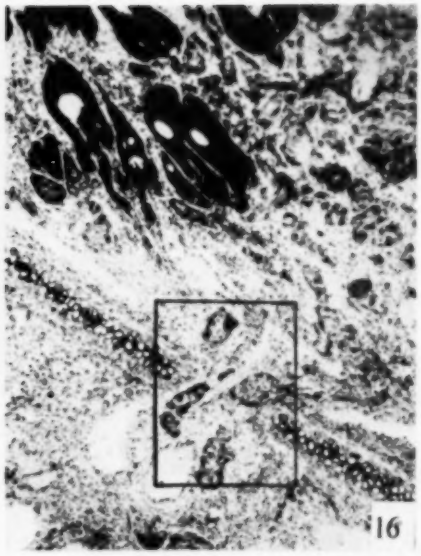
12


13

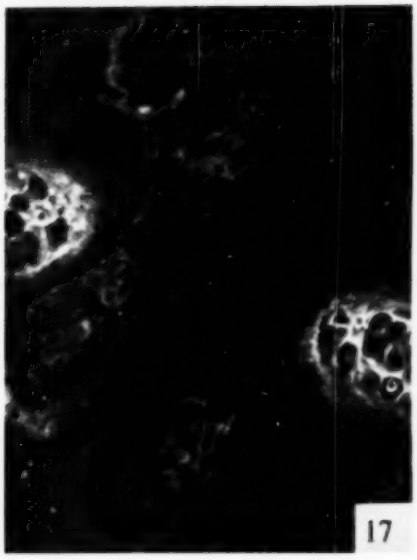

14

Louis.

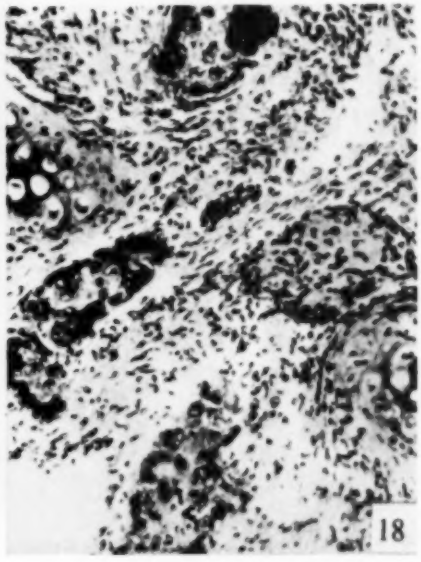
15



16



17



18

19

20

between viral and chemical carcinogens is becoming progressively more difficult to maintain and the present work supports the view that the virus is a variant of the chemical carcinogen rather than a qualitatively distinct agent. Thus the general fluorescence staining characteristics of malignant cells are remarkably constant and the results appear independent of any aetiological agent.

REFERENCES

Coons, A. H. and Kaplan, M. H.—(1950) *J. exp. Med.*, **91**, 1.

Creech, H. J. and Jones, R. N.—(1941*a*) *J. Amer. chem. Soc.*, **63**, 1661.—(1941*b*) *Ibid.* **63**, 1670.

Dineen, J. K. and Ada, G. L.—(1957) *Nature, Lond.*, **180**, 1284.

Eldredge, N. T. and Luck, J. M.—(1952) *Cancer Res.*, **12**, 801.

Heidelberger, C. and Moldenhauer, M. G.—(1957) *Ibid.*, **16**, 442.

Idem and Weiss, S. M.—(1951) *Ibid.*, **11**, 885.

Hopkins, S. J. and Wormall, A.—(1933) *Biochem. J.*, **27**, 740.

Hughes, P. E.—(1958) *Cancer Res.*, **18**, 426.

Idem and Louis, C. J.—(1959) *Arch. Path.*, **68**, 508.

Iidem, Dineen, J. K. and Spector, W. G.—(1957) *Nature, Lond.*, **180**, 289.

King, E. S. J., Hughes, P. E. and Louis, C. J.—(1958) *Brit. J. Cancer*, **12**, 5.—(1959) *Cancer*, **12**, 741.

De Lamirande, G., Allard, C. and Cantero, A.—(1953) *Ibid.*, **6**, 179.

Louis, C. J.—(1957*a*) *Stain Tech.*, **32**, 279.—(1957*b*) *Aust. N.Z. J. Surg.*, **27**, 146.—(1957*c*) *Aust. Ann. Med.*, **6**, 300.—(1957*d*) *Ibid.*, **6**, 277.—(1958*a*) *Brit. J. Surg.*, **46**, 147.—(1958*b*) *Surg. Gynec. Obstet.*, **107**, 317.—(1958*c*) *Brit. J. Cancer*, **12**, 537.—(1958*d*) *Aust. Ann. Med.*, **7**, 219.

Idem and Varasdi, G.—(1960) *Ann. Surg.* (in press).

Idem and White, J.—(1960) *Lab. Invest.*, **9**, 273.

Miller, E. C. and Miller, J. A.—(1947) *Cancer Res.*, **7**, 468.

Miller, J. A. and Miller, E. C.—(1952) *Ibid.*, **12**, 283.

Rogers, S. and Rous, P.—(1951) *J. exp. Med.*, **93**, 459.

Rous, P. and Beard, J. W.—(1935) *Proc. Soc. exp. Biol. N.Y.*, **32**, 578.

Shope, R. E.—(1933) *J. exp. Med.*, **58**, 607.

Sorof, S. and Cohen, P. P.—(1951) *Cancer Res.*, **11**, 376.

Smith, W. E., Kidd, J. G. and Rous, P.—(1952) *J. exp. Med.*, **95**, 299.

Syverton, J. T.—(1952) *Ann. N.Y. Acad. Sci.*, **54**, 1126.

RADIOACTIVE TRACER STUDIES OF RED CELL DESTRUCTION IN RATS BEARING A TRANSPLANTABLE TUMOUR

E. H. BELCHER AND SHIRLEY M. SIMPSON

From the Postgraduate Medical School, London, W.12

Received for publication April 2, 1960

ANAEMIA is frequently observed in cancer patients and also in animals bearing transplantable or induced tumours; this association is the subject of a recent review by Price and Greenfield (1958). It is probable that both decreased erythropoiesis and increased erythrocyte destruction contribute to the reduction in haemoglobin concentration, haematocrit value or red cell volume observed in anaemias associated with cancer. However, evidence has accumulated that an increased rate of destruction of the circulating red cells is common in human cancer. Thus Berlin (1951) demonstrated by the Ashby differential agglutination technique an increased rate of destruction of transfused normal cells in patients with myeloid or lymphatic leukaemia. An increased rate of destruction of the patient's own circulating red cells labelled with glycine-2-^{14}C was found by Berlin, Lawrence and Lee (1954) in leukaemia. Hyman, Gellhorn and Harvey (1956), using both the Ashby technique and the transfusion of cells labelled with ^{51}Cr found an increased rate of destruction of cells transfused from normal donors to patients with carcinomata and other neoplasms, whereas cells transfused from cancer patients to normal volunteers had a normal or near-normal life span. Hence they argued the absence of any intrinsic defect in the red cells of cancer patients. Miller, Chodos, Emerson and Ross (1956) also demonstrated by the Ashby and the ^{51}Cr-techniques an increased rate of destruction of red cells transfused from normal subjects to patients with cancer of various sites or autotransfused to such patients.

The results of studies on animals bearing transplantable or induced tumours have been more conflicting. Ultman, Fish and Hyman (1956), in studies of ^{51}Cr-survival in rabbits bearing the Brown-Pearce or XV2 carcinoma, failed to reveal any increased destruction of red cells except in animals with severe infection. Ehrenstein (1958) used glycine-2-^{14}C to study the life span of the circulating red cells in mice bearing the Ehrlich ascites carcinoma and found increased red cell destruction of the random type in tumour-bearing animals. Greenfield, Godfrey and Price (1958), using ^{59}Fe-labelled cells, also observed increased red cell destruction in rats bearing Lymphosarcoma 2788, Hepatoma 3683, the Yoshida ascites tumour and the Harderian gland Carcinoma 2226. They found that as red cell destruction proceeded, ^{59}Fe accumulated within the tumour. In all of these tumours, haemorrhage or thrombosis was observed. In the mouse Fibrosarcoma HE 8971 and Hepatoma 134 (ascites) and the rat Fibrosarcoma 4956, in which there was little or no haemorrhage or thrombosis, anaemia and loss of labelled red cells was small and did not occur until the late stages of tumour growth. In further studies by the same group, Greenfield, Sterling and Price (1958) transfused ^{51}Cr-labelled red cells to rats bearing the Lymphosarcoma R 2788 and were able

to recover from the tumour nearly all the ^{51}Cr lost from the blood ; they concluded that increased red cell destruction was the consequence of vascular injury in the neighbourhood of the tumour. Price, Greenfield, Sterling and MacCardle (1959) transfused mixtures of ^{51}Cr- and ^{59}Fe-labelled red cells to rats with Lymphosarcoma R 2788 and found that the subsequent distributions of the two isotopes within the tumour were similar, uptake being greatest in haemorrhagic areas, less in necrotic areas and almost negligible in white viable tumour. In this tumour, haemorrhage into the surrounding connective tissue is an important phenomenon. The extent of increased red cell destruction in other tumours could be correlated with their histological appearance. Thus in Fibrosarcoma 4956, little haemorrhage was observed and increased red cell destruction was not detected until the tumour had become massive and ulceration of the skin had occurred.

In the investigations described below, red cell destruction has been studied by the ^{51}Cr- and ^{59}Fe-techniques in rats of the " August " strain bearing the transplantable mammary adenocarcinoma R 2426, in which neither haemorrhage nor necrosis are observed. Particular interest was attached to this tumour since a haemolytic episode in the host animal had been observed to follow its implantation. Preliminary reports of these studies have already been published (Belcher, 1958, 1959).

MATERIALS AND METHODS

Rats.—Rats of the pure-line " August " strain of weight 100–200 g. were used throughout. They were maintained on Medical Research Council Diet No. 41 and water *ad libitum*. Frequent observations on splenectomised animals failed to reveal any latent *Bartonella muris* infection.

Transplantation of tumour.—Tumour R 2426 was transplanted at intervals of about 4 weeks. Implantation of tumour was carried subcutaneously under aseptic conditions through a trochar inserted into the left abdominal wall, the site of implantation being afterwards closed by a small suture clip. Four weeks after implantation, when transplantation was again due, the growing tumour was firm and round with a maximum diameter of about 2 cm. On sectioning, it was normally found to be white and viable throughout, with no evidence of necrotic or haemorrhagic areas.

Studies with ^{51}Cr-labelled red cells

Red cell survival studies with ^{51}Cr were made as described by Belcher and Harriss (1959). In order to reduce the possibility of uncontrolled transfusion reactions, transfusion of blood was carried out under aseptic conditions between male litter mates, one member of each litter acting as donor, the others as recipients. In studies with ^{51}Cr, the red cells of donor animals were labelled *in vitro* with ^{51}Cr as follows : 2 ml. of blood were withdrawn by cardiac puncture into a syringe containing 0·1 ml. heparin (" Pularin ", Evans Medical Supplies Ltd.), and delivered into a graduated test tube. One ml. of acid-citrate dextrose-solution and 1 ml. of a solution of sodium chromate—^{51}Cr in isotonic saline containing about 50 μc./ml. ^{51}Cr (specific activity about 10 μc./μg. Cr) were added. The mixture was allowed to stand for 30 minutes with occasional shaking. It was then gently centrifuged, the supernatant diluted plasma pipetted off and the cells resuspended in isotonic saline, the final volume being readjusted to 2 ml. The

labelled cell suspension was further diluted with isotonic saline if desired. Of the added ^{51}Cr, 90–95 per cent entered the red cells.

Portions of the labelled cell suspension 0·5 ml. in volume were injected without delay into litter mate recipients, injection being carried out either intravenously into a lateral tail vein or intraperitoneally. At daily intervals thereafter, the extreme tip of the tail of each recipient was cut under light ether anaethesia and a 0·02 ml. blood sample taken into a haemoglobin pipette and delivered into 5 ml. ammoniated distilled water for haemoglobin estimation. Haemoglobin was estimated colorimetrically as HbO_2 in the haemolysed blood samples. These were then transferred to $\frac{5}{8}$ inch diameter sample tubes and assayed for ^{51}Cr in a well scintillation counter coupled to a scaler through a linear amplifier and single channel pulse height discriminator/analyser. The ^{51}Cr concentrations in successive samples of blood were expressed as percentages of that of the sample taken from the same animal on the first day after transfusion and the result corrected for the increase in blood volume with increase in weight of the animal during the period of observation (Belcher and Harriss, 1957).

Studies of ^{51}Cr-distribution in the tissues were made as described by Belcher, Lamerton and Harriss (1958). Recipient animals were killed by cervical fracture under ether anaesthesia. They were then exsanguinated as completely as possible by opening the thorax, excising the left lung and injecting isotonic saline into the left ventricle of the heart, diluted blood being removed as it accumulated in the thoracic cavity. The carcasses were then carefully dissected and selected tissues removed to $\frac{5}{8}$ inch diameter sample tubes for ^{51}Cr-assay. Small corrections were applied for the variation in efficiency of the scintillation counter with sample volume.

Studies with ^{59}Fe-labelled red cells

Red cell survival studies with ^{59}Fe were made as described by Belcher and Harriss, 1959. The circulating red cells of donor animals were labelled *in vivo* with ^{59}Fe as follows ; 10 μc. ^{59}Fe (specific activity 1–5 μc./μg. Fe) as ferric chloride in 0·5 ml. 1 per cent w/v sodium citrate solution were injected intravenously to each donor. After an interval of 3 days to allow cells labelled in the erythropoietic tissues to emerge into the circulation, about 2 ml. of blood were withdrawn by cardiac puncture from each donor into a heparinised syringe and delivered into a graduated test tube. The ^{59}Fe-labelled red cells were further labelled *in vitro* with ^{51}Cr as described above if desired. Portions of labelled cell suspension 0·5 ml. in volume were then injected intravenously into litter mate recipients. Daily 0·02 ml. blood samples were taken from the cut tails of the recipient animals as already described, haemolysed in 5 ml. ammoniated distilled water and transferred to $\frac{5}{8}$ inch diameter sample tubes for radioactive assay. To suppress re-utilisation of ^{59}Fe from destroyed red cells by the erythropoietic tissues daily injections of 0·1 ml./100 g. body weight of an iron-dextran complex (" Imferon ", Bengers Laboratories Ltd.) containing 50 mg./ml. Fe were given intramuscularly throughout studies of red cell survival of ^{59}Fe-labelled cells.

In the radioactive assay of samples in single tracer experiments with ^{51}Cr or ^{59}Fe alone, the discriminator/analyser unit was operated as a simple discriminator. In double tracer experiments with ^{51}Cr and ^{59}Fe, samples were first assayed for ^{59}Fe by using the unit as a discriminator set at a level such that the system was almost completely insensitive to ^{51}Cr. Samples were then assayed for ^{51}Cr using

the unit as an analyser aligned on the peak of the ^{51}Cr gamma spectrum, a correction being applied for the small contribution to the total counting rate due to ^{59}Fe on the basis of the already determined ^{59}Fe content of the sample.

RESULTS

Response of " August " rats to implantation of tumour R 2426

August rats receiving subcutaneous implants of tumour R 2426 undergo an acute haemolytic episode between 10 and 15 days after implantation, at which time the growing tumour has the size of a small pea. During the episode, which is

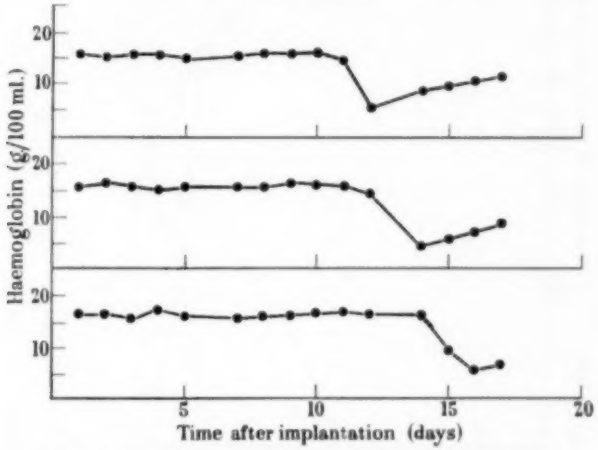


FIG. 1.—Blood haemoglobin in " August " rats receiving subcutaneous implants of tumour R 2426.

accompanied by haemoglobinuria and which may be fatal, the haemoglobin concentration of the circulating blood falls to about 5 g./100 ml. (Fig. 1). If the animal survives, the haemoglobin concentration returns to normal within a further 10 days.

Survival of red cells from normal donors labelled with ^{51}Cr and transfused to tumour-bearing recipients

If red cells from a normal donor are labelled with ^{51}Cr and 0·5 ml. of the labelled cell suspension transfused to a litter mate recipient animal which is then given a tumour implant, the fate of the circulating red cells during the haemolytic episode can be followed (Fig. 2). Up to the start of the haemolytic episode, red cell survival is within the normal limits quoted by Belcher and Harriss (1959), but during the episode almost all of the transfused cells are removed, the ^{51}Cr-concentration in the blood falling more steeply than the haemoglobin concentration which is to some extent maintained by compensatory erythropoiesis.

Red cells from normal donors labelled with ^{51}Cr and transfused to tumour-bearing recipients after implantation of tumour but before the haemolytic episode or during its development are similarly removed from the circulation during the episode (Fig. 3). However, if cells from normal donors are labelled with ^{51}Cr and

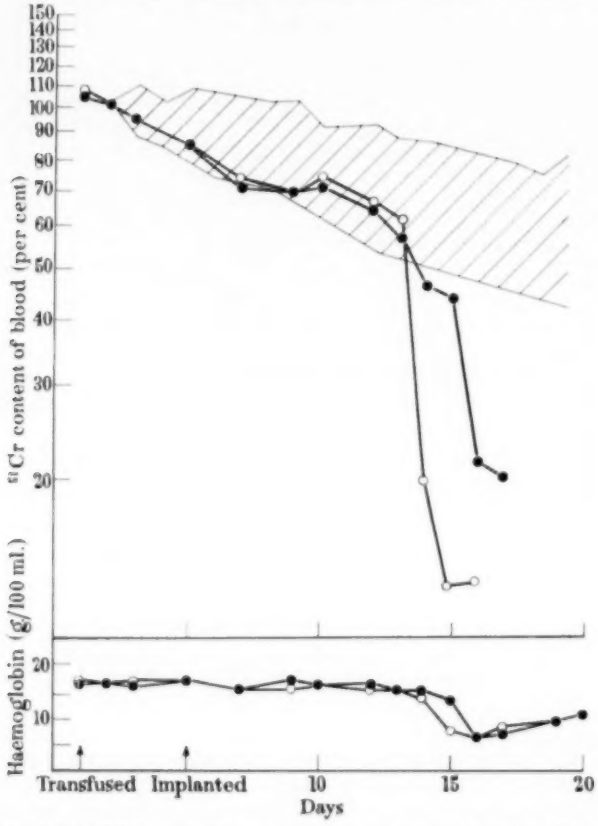


*Fig. 2.—Blood haemoglobin and ^{51}Cr-concentration in tumour-bearing "August" rats transfused with 0·5 ml. ^{51}Cr-labelled red cells from normal litter-mate donors 4 days after implantation with tumour R 2426.

Range of ^{51}Cr-concentration in normal "August" rats transfused with ^{51}Cr-labelled cells from litter-mate donors.

transfused to tumour-bearing recipients which have already suffered their haemolytic episode and are in recovery from it, the labelled cells are not rapidly removed, red cell destruction being only slightly, albeit significantly, more rapid than in normal animals (Fig. 4). At this time therefore, it appears either that the concentration of the haemolytic agent in the host animal is reduced or that some mechanism is operating to reduce its effect on the circulating red cells.

* In this and subsequent figures, except Fig. 11, the graph lines shown by solid and open points refer to two identically treated rats.

Survival of red cells from tumour-bearing donors labelled with ^{51}Cr and transfused to tumour-bearing recipients

Red cells from tumour-bearing donors labelled with ^{51}Cr and transfused to tumour-bearing recipients before or during the development of the haemolytic episode are rapidly removed during the episode, as are cells from normal donors.

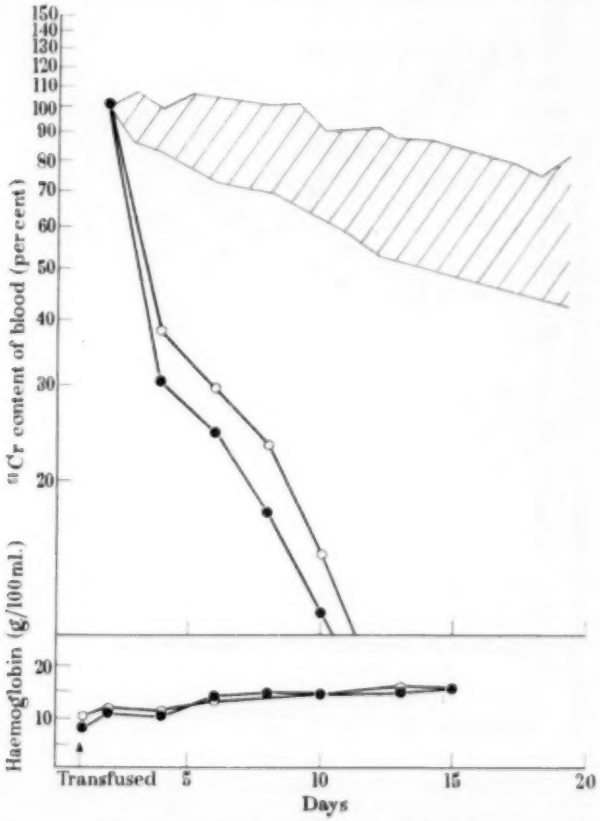


FIG. 3.—Blood haemoglobin and ^{51}Cr-concentration in tumour-bearing "August" rats transfused with 0·5 ml. ^{51}Cr-labelled red cells from normal litter-mate donors 13 days after implantation with tumour R 2426.

Range of ^{51}Cr-concentration in normal "August" rats transfused with ^{51}Cr-labelled cells from litter-mate donors.

When cells from tumour-bearing animals in recovery from their haemolytic episode are labelled and transfused to other tumour-bearing animals in recovery, red cell destruction appears within normal limits (Fig. 5). However, in this situation the transfused cells have a grossly abnormal age distribution with a preponderance of young cells, since most of the donor's circulating red cells were destroyed during the episode. Under these circumstances, the observation of apparently normal

^{51}Cr-survival indicates a slightly increased red cell destruction rate, such as is observed when cells are transfused from normal donors to tumour-bearing recipients in recovery.

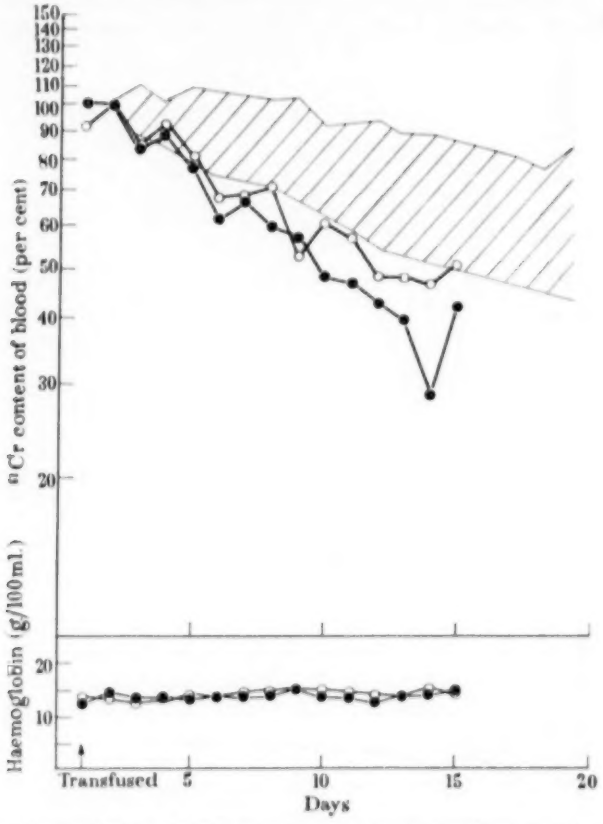


FIG. 4.—Blood haemoglobin and ^{51}Cr-concentration in tumour-bearing "August" rats transfused with 0·5 ml. ^{51}Cr-labelled red cells from normal litter-mate donors 14 days after implantation with tumour R 2426.

Range of ^{51}Cr-concentration in normal "August" rats transfused with ^{51}Cr-labelled cells from litter-mate donors.

Survival of red cells from tumour-bearing donors doubly labelled with ^{59}Fe and ^{51}Cr and transfused to tumour-bearing recipients

It is of interest whether red cells are destroyed during the haemolytic episode in a random fashion without regard to age, or whether older cells are more rapidly destroyed than younger ones. To study this question, a donor rat implanted with tumour 3 days previously was given an intravenous injection of 10 μc. ^{59}Fe. Three days later, when the ^{59}Fe-labelled red cell precursors had emerged into the circu-

lating blood, blood was withdrawn from the donor animal by cardiac puncture and labelled *in vitro* with ^{51}Cr. The labelled red cell suspension thus obtained contained cells of all ages labelled with ^{51}Cr and included a single cohort of young cells labelled with ^{59}Fe. This suspension was then used for survival studies in

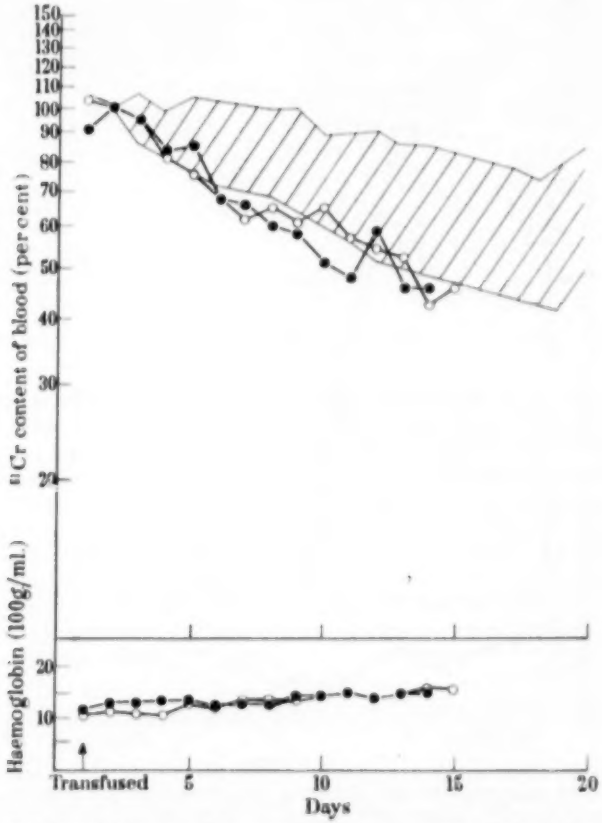


FIG. 5.—Blood haemoglobin and ^{51}Cr-concentration in tumour-bearing "August" rats transfused with 0·5 ml. ^{51}Cr-labelled red cells from tumour-bearing litter-mate donor 14 days after implantation with tumour R 2426.

Range of ^{51}Cr-concentration in normal "August" rats transfused with ^{51}Cr-labelled cells from litter-mate donors.

litter mate recipients (Fig. 6). Despite the daily injection of iron-dextran to block re-utilisation, some of the ^{59}Fe from destroyed red cells is incorporated into the haemoglobin of red cell precursors in the erythropoietic tissues and thus returned to the circulation during the haemolytic episode. For this, reason, the ^{59}Fe-survival curve falls less rapidly than does that for ^{51}Cr. Apart from this observation, how-

ever, no significant difference can be observed between the survival curves for ^{51}Cr and ^{59}Fe-labelled red cells during the episode. It thus appears that cells are destroyed at random, without regard to age, during this episode.

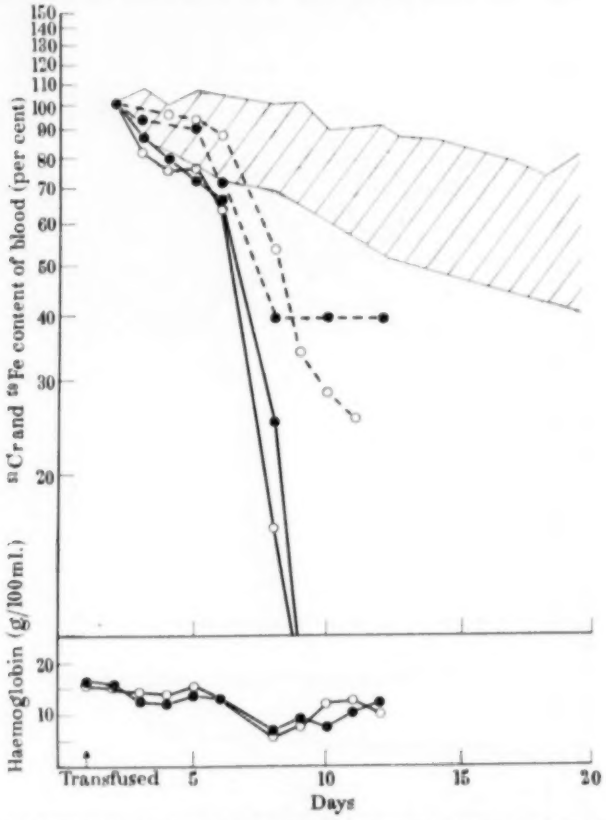


FIG 6.—Blood haemoglobin, ^{51}Cr and ^{59}Fe levels in tumour-bearing "August" rats given 0·5 ml. of red cells doubly labelled with ^{51}Cr and ^{59}Fe from tumour-bearing litter-mate donor 6 days after implantation with tumour R 2426.

○- - - -○ ^{59}Fe content. ○——○ ^{51}Cr content.

▭ Range of ^{51}Cr-concentration in normal "August" rats transfused with ^{51}Cr-labelled cells from litter-mate donors.

Sites of destruction of red cells from tumour-bearing donors labelled with ^{51}Cr and transfused to tumour-bearing recipients

To identify the chief sites of red cell destruction during the haemolytic episode, red cells from tumour-bearing donors were labelled with ^{51}Cr and transfused to tumour-bearing recipients before the development of haemolysis. During or after the subsequent episode, recipient animals were sacrificed and exsanguinated.

TABLE I.—*Distribution of ^{51}Cr in Tissues and Excreta of Four "August" Rats Bearing Tumour R 2426, Transfused with ^{51}Cr-labelled Cells from Normal Litter-mate Donors 5–6 days After Implantation of Tumour and Sacrificed During or After Subsequent Haemolytic Episode.*

Interval between implantation and transfusion (days)	5		6	
Interval between implantation and assay (days)	11		15	
Tissue	^{51}Cr content (% injected dose)		^{51}Cr content (% injected dose)	
Blood	18·0	17·0	13·0	14·4
Spleen	15·0	10·0	12·0	13·5
Liver	27·0	23·0	12·0	27·0
Kidneys	5·0	2·5	1·5	5·5
Lungs	1·5	0·8	0·4	1·0
Tumour	0·1	0·2	0·1	0·1
Other tissues	17·3	17·8	5·4	8·0
Total urine	4·5	14·0	25·0	23·7
Total faeces	11·0	8·0	14·0	6·2
Recovery	99·4	93·3	93·4	99·4

Selected tissues were then dissected out and assayed for ^{51}Cr. Results are summarised in Table I. It can be seen that ^{51}Cr accumulates in both the liver and the spleen, the highest concentration, but not necessarily the greatest amount being found in the spleen. In contrast to the observations of Greenfield, Sterling and Price (1958), uptake in the tumour is negligible or extremely small. The livers and spleens of animals sacrificed after their haemolytic episode are found to be enlarged and engorged with blood. It thus appears that the chief sites of red cell destruction during the episode are the liver and spleen, which together account for some 40 per cent of the radioactivity lost from the circulating blood. Red cell destruction due to haemorrhage in the vascular bed of the tumour is absent or extremely small.

Survival of red cells from tumour-bearing donors labelled with ^{51}Cr and transfused to normal recipients

August rats receiving transfusions of blood from donor animals bearing tumour R 2426 undergo a haemolytic episode similar to that observed in animals receiving implants of tumour. Fig. 7–10 show the results of experiments in which blood was taken from tumour-bearing donors at 4, 9, 14 and 19 days after implantation of tumour and labelled with ^{51}Cr; 0·5 ml. of the labelled red cell suspension or of the supernatant plasma was then transfused intravenously to normal litter mate recipients. When transfusion of red cells is carried out at 4 days after implantation, at which time the growing tumour is scarcely palpable (Fig. 7), the haemolytic episode is preceded by a period of normal survival of the labelled cells. During the episode, both the transfused cells and the donor's own circulating red cells are destroyed simultaneously. Cells transfused at 9 days, shortly before the donor's own haemolytic episode (Fig. 8), cause a more prompt haemolysis, though a period of normal survival can still be seen. At 14 days (Fig. 9), haemolysis in the recipient is almost immediate and frequently fatal.

When transfusion of cells is carried out at 19 days after implantation, however, when the donor is in recovery (Fig. 10), a different pattern is observed ; a haemolytic episode takes place in the recipient about 4 days after transfusion, but this episode affects only the recipient's own cells and the survival of the transfused labelled cells appears only slightly less than normal. It must again be emphasised that since the age distribution of these labelled cells is abnormal, with a preponderance of young cells, the observation of a nearly normal ^{51}Cr-survival indicates that the labelled cells in fact have a shortened life span. Nevertheless, it is

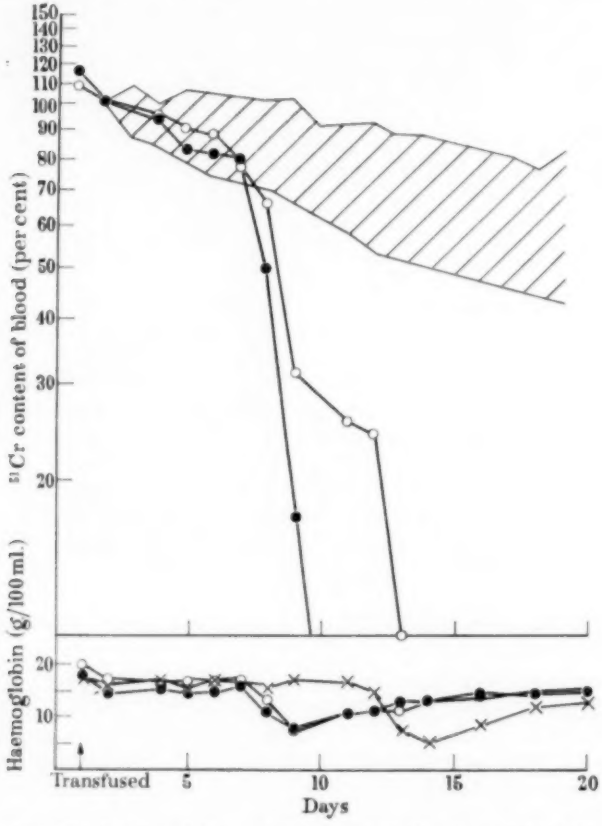


FIG. 7, 8, 9, 10.—Blood haemoglobin and ^{51}Cr-concentration in normal "August" rats transfused with 0·5 ml. ^{51}Cr-labelled red cells or plasma from litter-mate donors bearing tumour R 2426.

○ Recipients receiving labelled red cell suspension.
× Recipients receiving diluted plasma.

FIG. 7.—Blood taken from donor 4 days after implantation.

▨ Range of ^{51}Cr-concentration in normal "August" rats transfused with ^{51}Cr-labelled cells from litter-mate donors.

clear that the donor's cells are not involved to any great extent in the haemolytic episode which destroys the recipient's cells.

Transfusion of plasma in all cases caused a haemolytic episode in the recipient similar to that following transfusion of red cells but occurring about 4 days later in time.

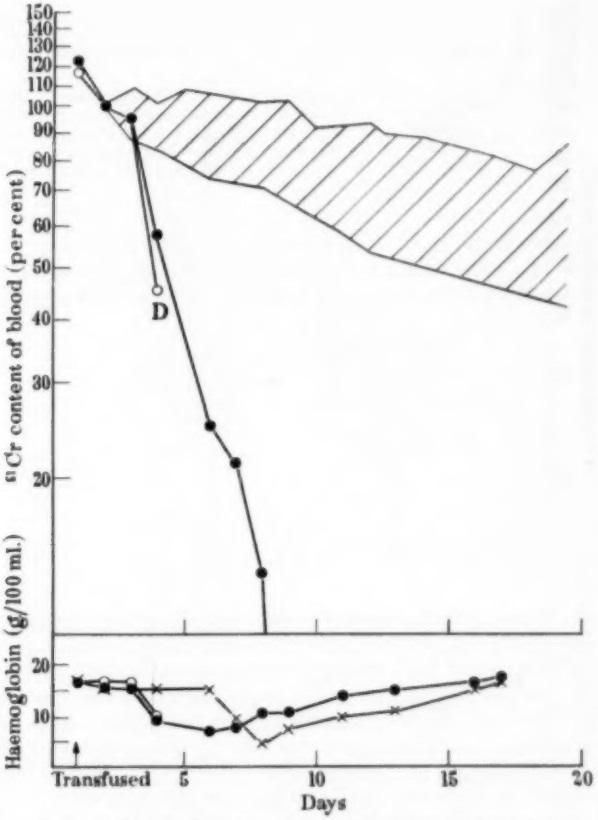


FIG. 8.—Blood taken from donor 9 days after implantation.

▨ Range of ^{51}Cr-concentration in normal "August" rats transfused with ^{51}Cr-labelled cells from litter-mate donors.

Fig. 11 shows the results of an experiment comparing the effects of intravenous and intraperitoneal transfusion of ^{51}Cr-labelled cells from tumour-bearing donors to normal recipients. Intraperitoneally injected ^{51}Cr-labelled cells rapidly appear in the circulation and their haemolytic activity does not appear to be decreased by passage through the peritoneal cavity, since no difference may be observed between the survival of labelled cells in animals transfused intravenously and that that in animals transfused intraperitoneally.

In further experiments, blood was taken from a tumour-bearing animal shortly before it was due to undergo its haemolytic episode and the labelled red cell suspension diluted with isotonic saline. 0·5 ml. portions of the diluted suspension were then transfused intravenously to normal recipients and the interval between transfusion and the onset of haemolysis in the recipient measured. Results are

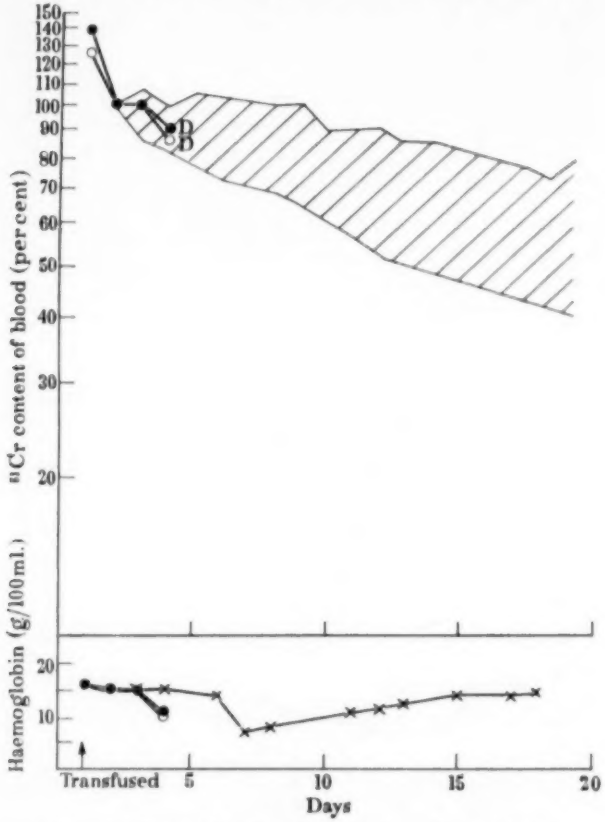


FIG. 9.—Blood taken from donor 14 days after implantation.

▨ Range of ^{51}Cr-concentration in normal "August" rats transfused with ^{51}Cr-labelled cells from litter-mate donors.

summarised in Table II. With dilution, the haemolytic activity of the donor's blood is progressively reduced, as measured by the interval between transfusion and the onset of haemolysis, but even at a dilution of ×1000, a haemolytic episode is still observed. This dilution corresponds to the transfusion of only 0·0005 ml. of blood.

The data of Fig. 7–10 may now be reconsidered in the light of these dilution studies. Table III summarises the results of those experiments in which 0·5 ml.

TABLE II.—*Interval Between Transfusion and Onset of Haemolytic Episode in "August" Rats Transfused with 0·5 ml. Washed Red Cell Suspension Taken from Litter-mate Donors 14 days After Implantation with Tumour R 2426*

Material transfused	Dilution	Interval between transfusion and onset of haemolytic episode (days)
Saline	1	No episode
Suspension of red cells from non-tumour-bearing donor	1	"
Suspension of red cells from tumour-bearing donor	1	3
Suspension of red cells from tumour-bearing donor	0·1	5
Suspension of red cells from tumour-bearing donor	0·01	7
Suspension of red cells from tumour-bearing donor	0·001	9

TABLE III.—*Interval Between Transfusion and Onset of Haemolytic Episode in "August" Rats Transfused with 0·5 ml. Washed Red Cell Suspension Taken from Litter-mate Donors at Different Times after Implantation with Tumour R 2426*

Interval between implantation and transfusion (days)	Interval between transfusion and onset of haemolytic episode (days)
1	No episode
3	12
4	9
9	3
14	2
19	4
35	10

of labelled red cells was transfused from tumour-bearing donors to normal recipients at different times after implantation of the donors. The haemolytic activity of the donor's blood as measured by the rapidity with which it promotes a haemolytic episode increases to a maximum which is reached during or just after the episode in the donor, and then slowly declines. Whilst the results of experiments involving different donors are not strictly comparable, an approximate evaluation of the relative haemolytic activity of the donor's blood at different times after implantation may be made by comparison with the data of Table II.

The relative effects of injections of red cells and of plasma which were reported in Fig. 7–10 may be similarly evaluated. The results show that the haemolytic agent is mainly associated with the cellular fraction and is only found in the plasma at a concentration lower by at least two orders of magnitude.

The effect of multiple transfusions of red cells from tumour-bearing donors to normal recipients

Following the induction of a haemolytic episode in an animal receiving a tumour implant or a transfusion from a tumour-bearing animal, it has not been found possible to induce a second episode either by transfusion or implant. Fig. 12 shows the results of an experiment in which labelled cells were transfused to normal

recipients from a tumour-bearing litter mate donor during its haemolytic episode and caused the expected haemolytic response. Four weeks later, the recipients were transfused with ^{51}Cr-labelled red cells from a second tumour-bearing litter mate donor also undergoing its haemolytic response. On this occasion, no haemolytic episode is observed, although the same red cell suspension injected into the

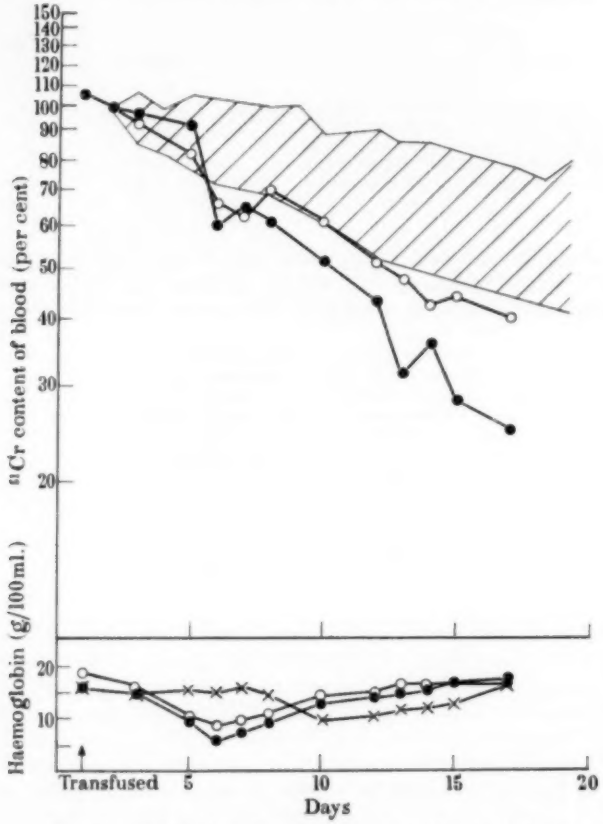


FIG. 10.—Blood taken from donor 19 days after implantation.

Range of ^{51}Cr-concentration in normal "August" rats transfused with ^{51}Cr-labelled cells from litter-mate donors.

animals previously transfused did not fail to produce the expected pattern of haemolysis.

Such protection has been found to extend at least up to two months after the initial haemolytic response. It has already been described how labelled red cells transfused from a tumour-bearing donor which has undergone its haemolytic episode to a normal recipient have a normal or near-normal survival (Fig. 10). although a haemolytic episode is induced in the recipient. This is a further example

of the protection afforded to the red cells of an animal that has undergone a haemolytic episode.

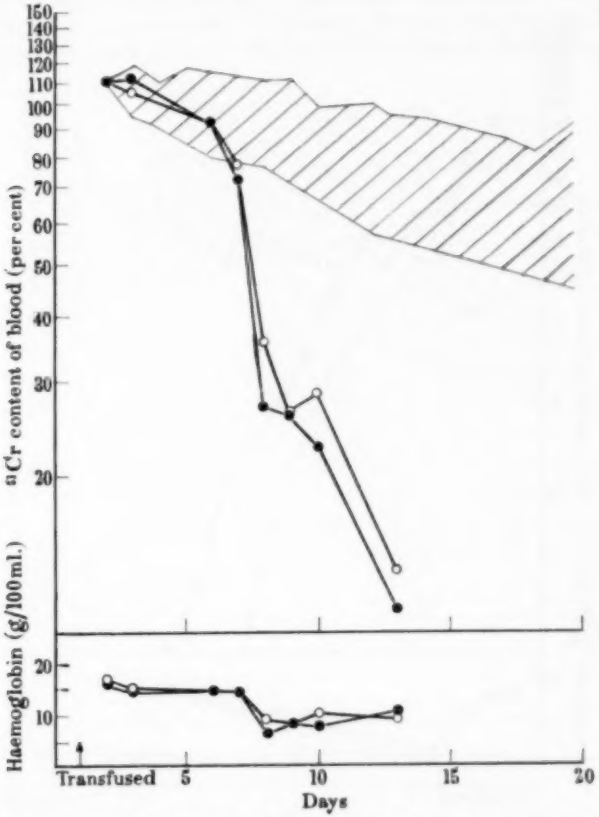


FIG. 11.—Blood haemoglobin and ^{51}Cr-concentration in normal "August" rats transfused with 0·5 ml. ^{51}Cr-labelled red cells from tumour-bearing litter-mate donor 4 days after implantation of latter with tumour R 2426.

○ Injection by intraperitoneal route.
● Injection by intravenous route.

▨ Range of ^{51}Cr-concentration in normal "August" rats transfused with ^{51}Cr-labelled cells from litter-mate donors.

DISCUSSION

In the absence of the results of serological studies which are still incomplete, detailed discussion of the mechanisms underlying the phenomena described must be speculative. However, it is clear that the haemolytic episode and anaemia observed after implantation of the tumour R 2426 do not resemble that described by Greenfield and his co-workers, since no accumulation of ^{51}Cr in the tumour is observed after transfusion of ^{51}Cr-labelled red cells.

The haemolytic episode has some features in common with those observed in auto-immune haemolytic anaemias attributed to the presence of incomplete warm agglutinins (Dacie, 1954; Jandl, Jones and Castle, 1957), notably the accumula-

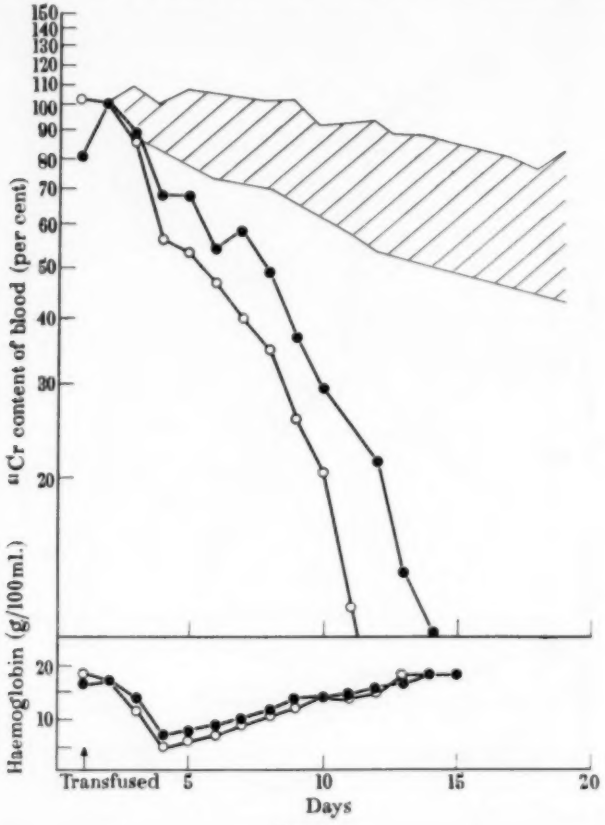


FIG. 12A.

FIG. 12.—Blood haemoglobin and ^{51}Cr-concentration in normal "August" rats transfused on two occasions at an interval of 4 weeks with 0·5 ml. ^{51}Cr-labelled red cells from tumour-bearing litter-mate donors 10 days after implantation of latter with tumour R 2426.

A Response to first transfusion.
B Response to second transfusion.

▨ Range of ^{51}Cr-concentration in normal "August" rats transfused with ^{51}Cr-labelled cells from litter-mate donors.

tion of ^{51}Cr on the liver and spleen during the episode. Such agglutinins might be evoked in response to the tumour graft in a manner similar to that in which antibodies to tumour or skin homografts which may be titrated as haemagglutinins are produced in mice (Hildeman and Medawar, 1959). Alternatively, they might be produced by the tumour itself against the host animal's cells as suggested

by Green, Wakefield and Littlerwood (1958). In either of these situations, the induction of haemolysis in a normal animal receiving a transfusion of blood from a tumour-bearing donor would be due to the transfer of antibody from the donor's red cells to those of the recipient. However, it has been demonstrated that a haemolytic episode can follow the transfusion of as little as 0·005 ml. of blood and

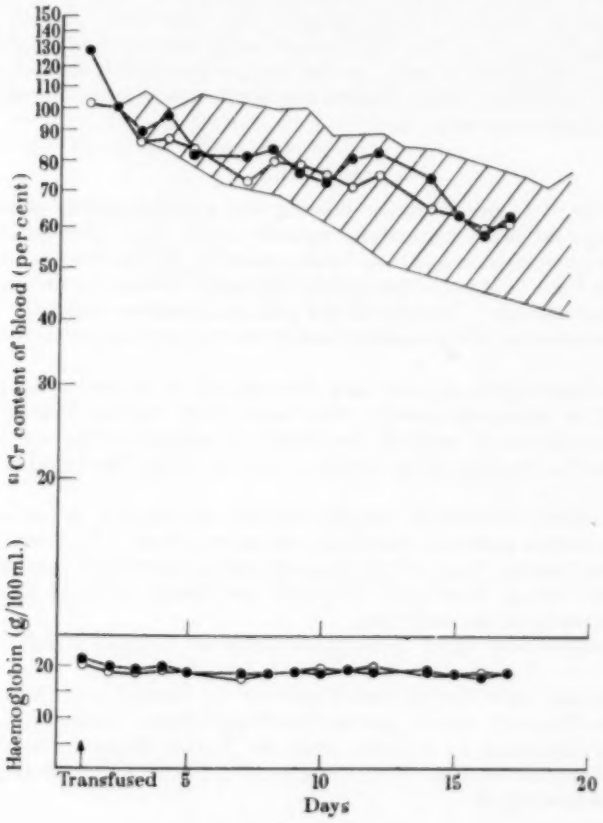


FIG. 12B.

it appears improbable that sufficient antibody could be transferred by such a mechanism to cause the haemolysis of nearly all the host's cells.

A further possibility is that the tumour might produce an antigen similar or complementary to antigenic components on the host's red cells. Antibodies produced by the host against this antigen might then induce agglutination of the red cells. The antigen could be transferred with the blood of a tumour-bearing donor to a normal recipient and induce a similar production of antibody and agglutination of the recipient's red cells. However, the finding that the time between transfusion

and onset of the haemolytic episode may be as little as two days does not seem to be compatible with such a mechanism.

The observations that the haemolytic activity of the blood increases steadily with time after implantation of tumour and that a haemolytic episode can follow the transfusion of minute amounts of blood from a tumour-bearing donor to a normal recipient suggest that the responsible agent is some factor, possibly viral in nature, which is capable of multiplication within the host. If such is the case, the question must be asked whether the virus is the causative agent of the tumour or whether it exists concomitantly in the tumour tissue but without aetiological relationship to it (Zilber, 1958). Further studies designed to test all of these possibilities are being carried out.

SUMMARY

Rats of the "August" strain bearing the transplantable adenocarcinoma R 2426 undergo an acute haemolytic episode 10–15 days after implantation of tumour. This phenomenon has been investigated by radioactive tracer techniques with ^{51}Cr and ^{59}Fe. Cells are destroyed randomly without regard to age during the haemolytic episode. Destroyed red cells accumulate primarily in the liver and spleen, destruction in the vascular bed of the tumour being absent or extremely small.

A similar haemolytic episode may be induced in normal animals transfused intravenously or intraperitoneally, with blood from tumour-bearing litter-mate donors. The rapidity of onset of the episode is related to the amount of blood transfused and to the time after implantation at which the blood is taken from the donor.

After an initial haemolytic episode induced by implant or transfusion, it is impossible to induce a second episode in the same animal; furthermore, red cells from a tumour-bearing donor which has suffered a haemolytic episode show near-normal survival when transfused to normal recipients, although they provoke a haemolytic episode in the recipient.

The mechanisms by which these phenomena are brought about are discussed.

This work has been carried out in part at the Institute of Cancer Research, Royal Cancer Hospital, and in part at the Postgraduate Medical School, and has latterly been supported by a grant from the British Empire Cancer Campaign. The technical assistance of Mr. H. C. Christie, Miss Frances Holmes and Miss Judith Topper is acknowledged.

REFERENCES

Belcher, E. H.—(1958) 3rd International Symposium on Radioactive Isotopes in Clinical Medicine and Research, Bad Gastein, Munich (Urban and Schwarzenberg), p. 206.—(1959) *Acta Un. int. Cancr*, **15**, 866.

Idem and Harriss, E. B.—(1957) *J. Physiol.*, **139**, 14.—(1959) *Ibid.*, **146**, 219.

Idem, Lamerton, L. F. and Harriss, E. B.—(1958) *Brit. J. Haemat.*, **4**, 390.

Berlin, N. I., Lawrence, J. H. and Lee, H. C.—(1954) *J. Lab. clin. Med.*, **44**, 860.

Berlin, R.—(1951) *Acta med. scand.*, **139**, Suppl. 252, p. 1.

Dacies, J. V.—(1954) 'The Haemolytic Anaemias, Congenital and Acquired'. London (Churchill).

EHRENSTEIN, G.—(1958) *Acta physiol. scand.*, **44**, 80.
GREEN, H. N., WAKEFIELD, JUNE AND LITTLERWOOD, G.—(1957) *Brit. med. J.*, ii, 779.
GREENFIELD, R. E., GODFREY, J. E. AND PRICE, V. E.—(1958) *J. nat. Cancer Inst.*, **21**, 641.
Idem, STERLING, W. R. AND PRICE, V. E.—(1958) *Ibid.*, **21**, 1099.
HILDEMAN, W. H. AND MEDAWAR, P. B.—(1959) *Immunology*, **2**, 44.
HYMAN, G. A., GELLHORN, A. AND HARVEY, J. L.—(1956) *Blood*, **9**, 618.
JANDL, J. H., JONES, A. R. AND CASTLE, W. B.—(1957) *J. clin. Invest.*, **36**, 1428.
MILLER, A., CHODOS, R. B., EMERSON, C. P. AND ROSS, J. F.—(1956) *Ibid.*, **35**, 1248.
PRICE, V. E. AND GREENFIELD, R. E.—(1958) *Advanc. Cancer Res.*, **5**, 199.
Iidem, STERLING, W. R. AND MACCARDLE, R. C.—(1959) *J. nat. Cancer Inst.*, **22**, 877.
ULTMANN, J. E., FISH, W. AND HYMAN, G. A.—(1956) *Cancer Res.*, **16**, 885.
ZILBER, L. A.—(1956) *Advanc. Cancer Res.*, **5**, 291.

STUDIES ON MOUSE LEUKAEMIA. THE FATE OF THYMUS HOMOGRAFTS IN IMMUNOLOGICALLY TOLERANT MICE

J. F. A. P. MILLER

From the Chester Beatty Research Institute, Institute of Cancer Research : Royal Cancer Hospital, Fulham Road, London, S.W.3

Received for publication March 28, 1960

NORMAL tissues have been successfully transplanted in foreign hosts which have been treated in such a way as to render them tolerant. Such tissues include skin (Billingham, Brent and Medawar, 1953), thyroid (Woodruff and Boswell, 1954 ; Woodruff and Sparrow, 1958), ovaries (Krohn, 1958 ; Martinez, Smith and Good, 1958), adrenals (Medawar and Russell, 1958), and pituitaries (Martinez *et al.*, 1958). The homotransplanted glands retain their functional integrity in the immunologically tolerant hosts : thyroid homografts are able to concentrate radio-iodine, adrenal homografts can sustain life in adrenalectomized animals on a salt-deficient diet, ovarian homografts give rise to sexual cycles and, when orthotopically transplanted, can produce ova from which litters eventually develop, and pituitary homografts can maintain normal growth and normal control over other endocrine glands.

Acquired tolerance of homografts of normal thymuses has been achieved in the experiments reported here by the intravenous inoculation during the neonatal period of living spleen or thymus cells taken from adult mice of the same strain as the prospective donors of the thymuses. It is now well established that lymphocytic leukaemia, whether spontaneous or induced, does not usually develop in the absence of thymus tissue (McEndy, Boon and Furth, 1944 ; Kaplan, 1950 ; Law and Miller, 1950*a*, *b* ; Gross, 1959 ; Levinthal, Buffett and Furth, 1959 ; Miller, 1959*a*, *b* ; 1960*b*) and that thymus grafting restores the potentiality for leukaemia development in isologous combinations (Law and Miller, 1950*a*, *b* ; Kaplan and Brown, 1954 ; Miller, 1959*b*, 1960*b*). It will be shown in this paper that thymus tissue from genetically susceptible mice can undergo malignant transformation in a foreign but tolerant host, and that a number of such malignant thymuses can be made to regress completely following the inoculation of activated immunologically competent cells.

MATERIALS AND METHODS

Mice.—Mice of the C3H/PW or C3Hf/PW strain, inbred in our laboratory since their acquisition from Bittner in 1938, show an incidence of spontaneous lymphocytic leukaemia lower than 5 per cent after 15 months of age. Spontaneous tumours of other types are also very rare, except in C3H female mice, over 80 per cent of which usually develop mammary tumours after 8 months.

The Ak_i strain, originally obtained from Dr. J. Furth via Professor J. Engelbreth-Holm, has been inbred here since 1945 and shows a high incidence of

spontaneous lymphocytic leukaemia, about 90 per cent of the mice developing the disease at approximately 9 months of age (Miller, 1960*a*).

Induction of immunological tolerance.—Cell suspensions from thymus or spleen of one-month-old Ak or C3H donors were prepared by teasing out the organ in buffered Ringer phosphate solution, washing twice, and resuspending to the volume at which 0·05 ml. of the suspension contained 5 to 8 million cells. This amount was injected intravenously into new-born C3H or Ak mice, respectively, less than 20 hours after birth. The technique of injecting the orbital branch of the anterior facial vein or the sigmoid sinus of the new-born mouse has been described and illustrated in the papers of Billingham and Brent (1956, 1959) and in a recent article by Brent (1959).

Marrow was expelled from the shafts of the femurs with a jet of Ringer phosphate through a No. 14 gauge needle mounted on a syringe. Gentle agitation by suction in and out of a pipette allowed the cells to separate from one another. They were then washed twice and resuspended so that each new-born mouse received about 8 million cells.

Thymectomy and thymus grafting.—Thymectomy was performed at 3 to 5 weeks of age as described previously (Miller, 1960*b*). Each thymectomized mouse was given a subcutaneous graft of a whole thymus from an untreated new-born C3H or Ak female mouse, as required, on the day of thymectomy. Thymuses from new-born mice were removed aseptically and introduced by a sterile trocar into the subcutaneous tissues of the right (C3H thymuses) or left (Ak thymuses) axilla. The mice were thereupon given 3000 to 4000 units of penicillin and 3 to 5 mg. of streptomycin daily for about 10 days to guard against infection.

Skin grafts.—Skin grafts from 1- to 2-month-old female Ak or C3H mice were transplanted to 6- to 8-week-old C3H or Ak mice by the method of Billingham and Medawar (1951) to provide an external indicator of the tolerant state.

Adoptive immunization.—The immune state may be acquired in three ways: (1) actively, by the introduction of an antigen into the animal, (2) passively, by the introduction of antibody prepared in another animal, and (3) by the transfer of immunologically activated cells from one animal to the other. Billingham, Brent and Medawar (1954) have named the state of immunity acquired in this third way "adoptive" immunity.

Normal 2-month-old C3H female mice were immunized against normal Ak tissues. Each C3H mouse was given bilateral skin grafts and an intraperitoneal injection of cells from two thymuses and two spleens from 1- to 2-month-old healthy Ak donors. Ten to eleven days later, at a time when the reaction in the skin graft was most intense, the mice were killed, and cell suspensions were prepared from their axillary and inguinal lymph nodes and spleen. The cells were immediately injected intraperitoneally into C3H mice tolerant of Ak to abrogate tolerance. Each tolerant host received cells from two spleens and twelve regional lymph nodes. These injections were repeated at intervals of 5 to 7 days as often as required.

Passage A filtrate.—This most powerful leukaemogenic filtrate was prepared from leukaemic mice of the C3Hf/Gs strain as described previously (Miller, 1960*a*). It was injected intraperitoneally (0·2 to 0·3 c.c.) into 3- to 5-day-old mice, as required, the needle first traversing the thigh muscles to avoid leakage.

Transplantation of tumours.—Cell suspensions were prepared from leukaemic spleens and made up in saline so that 0·5 ml. of the suspension contained 30 to 50

million cells. This was injected intraperitoneally into untreated adult C3H and Ak mice. Small pieces of leukaemic thymus were introduced aseptically by trocar under the skin in some of the mice.

Histology.—Sections were fixed in Bouin's fluid and stained in haematoxylin and eosin or other stains when indicated.

EXPERIMENTAL

Experiments were performed on both Ak mice tolerant of C3H and on C3H mice tolerant of Ak. The former were divided into three groups according to the nature of the cells injected intravenously at birth. Group 1 received C3H or C3Hf thymus cells, group 2, C3H spleen cells and group 3, C3H marrow cells. Some of the mice in group 1 also received an injection of Passage A filtrate, usually 3 to 5 days after birth. When inoculated as late as 14 days after birth (Miller, 1960*a*) this filtrate causes leukaemia to develop within 3 to 6 months in 100 per cent of non-thymectomized mice of the Ak strain (Miller, 1960*a*). All the mice were thymectomized at 3 to 5 weeks of age. In group 1, they were grafted subcutaneously with a day-old C3H or C3Hf thymus. In groups 2 and 3 they received isologous thymus grafts.

C3H mice made tolerant of Ak were divided into two groups. Mice in both groups were thymectomized but those in group 1 were grafted with day-old Ak thymuses while those in group 2 were grafted with day-old C3H thymuses.

EXPLANATION OF PLATES

FIG. 1.—Normal subcutaneous C3H thymus graft in an Ak mouse tolerant of C3H. This section was made 60 days after grafting.

FIG. 2.—Mammary adenocarcinoma in an Ak female mouse which received C3H marrow cells at birth.

FIG. 3.—Malignancy in a subcutaneous Ak thymus grafted to a tolerant C3H mouse.

FIG. 4.—High power view of leukaemic lymphocytes in thymus graft seen in Fig. 3. Note numerous mitoses.

FIG. 5.—Normal salivary gland tissue separated by a thin connective tissue capsule from the parotid gland tumour. Note the resemblance between the normal salivary ducts and the duct-like elements of the tumour.

FIG. 6.—Another salivary gland tumour showing clearly the duct-like pattern plus the loose mesenchymal elements.

FIG. 7.—A more solid type of salivary gland tumour. Note the adenomatous pattern merging into a confluent mass of cells.

FIG. 8.—High power view of a salivary gland tumour. Numerous mitotic figures are seen. The cells still show some grouping into glandular elements.

FIG. 9.—A pleomorphic sarcoma. Note the tumour giant cells and undifferentiated pattern of this tumour.

FIG. 10.—High power view of sarcoma seen in Fig. 9 to emphasize the nuclear variation and numerous mitoses.

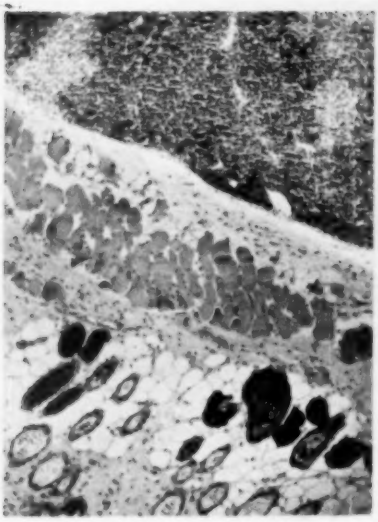
FIG. 11.—A fibrosarcoma infiltrating skeletal muscle.

FIG. 12.—A tumour from the upper eyelid. Note the well-differentiated pattern and bundles of uniform cells.

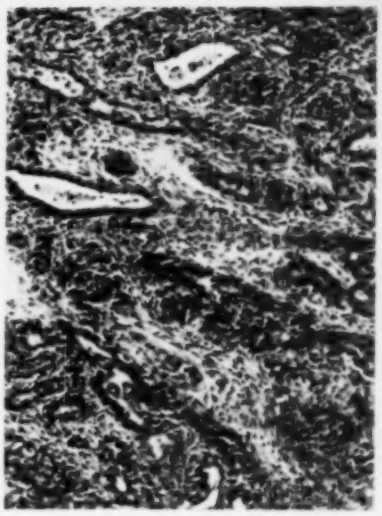
FIG. 13.—A necrotic anaplastic carcinoma. The grouping of the cells into clumps and the degenerative changes are clearly shown.

FIG. 14.—A kidney from a mouse with bilateral parotid tumours. Note the irregular lymphocytic infiltration in the cortex.

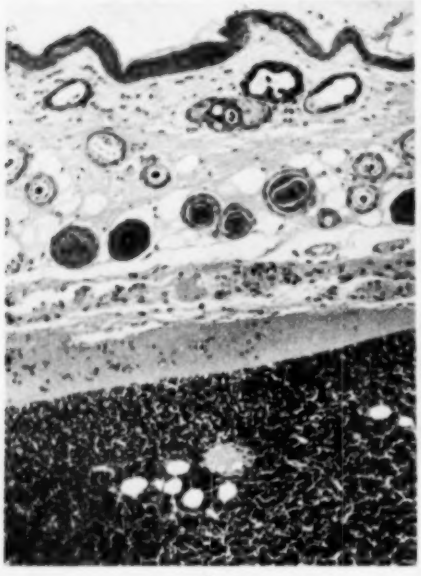
FIG. 15.—High power view of kidney shown in Fig. 14. Note the normal small lymphocytes grouped around blood vessels and the absence of mitoses.



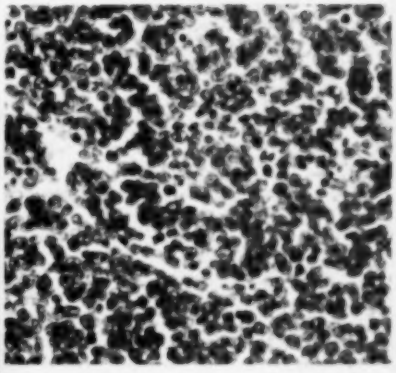
1



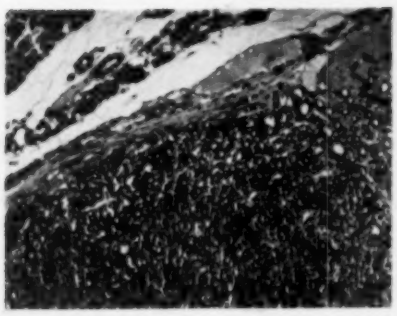
2



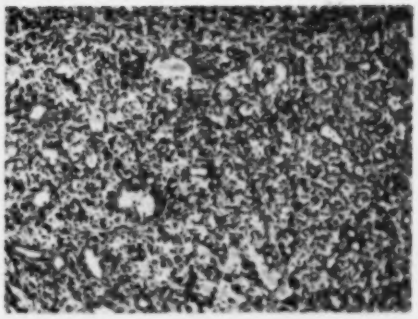
3



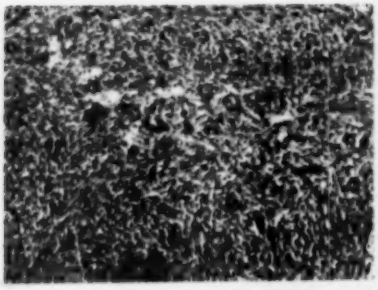
4



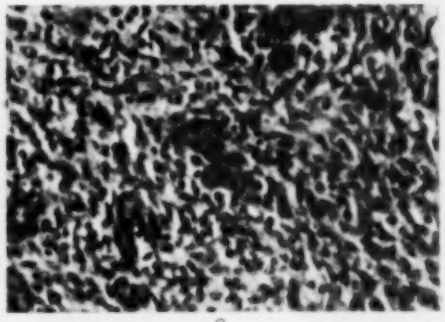
5



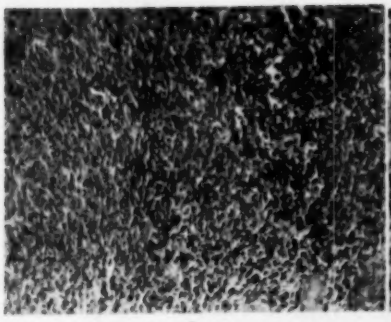
6



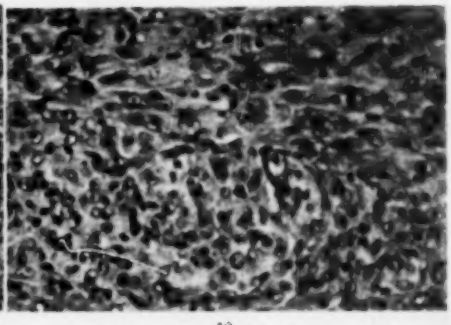
7



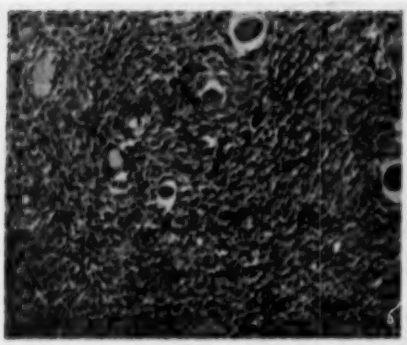
8



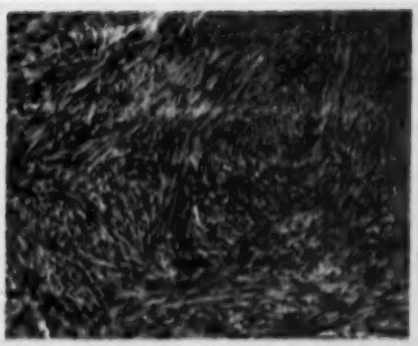
9



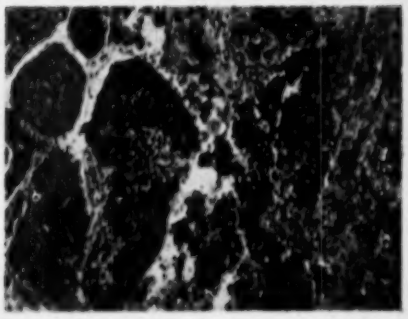
10



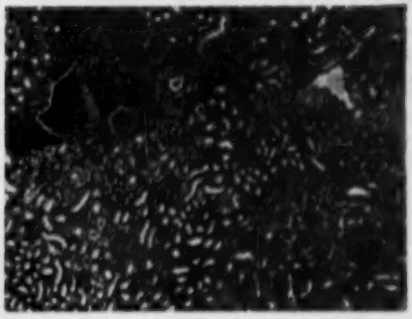
11



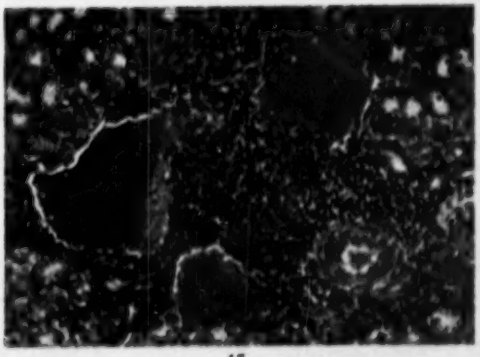
12



13



14



15

RESULTS

The results are presented in Tables I to V.

Induction of immunological tolerance in C3H or Ak mice

Tolerance of Ak skin grafts in C3H mice injected at birth with Ak spleen or thymus cells has already been described (Miller, 1960*a*) and the results are repeated here for comparison with tolerance of C3H skin grafts in the Ak strain. Up to 90 per cent of C3H mice were tolerant for periods up to a year or more. There was no evidence that spleen cells were more effective than thymus cells in inducing tolerance as they appeared to be in the strain combination used by Billingham and Brent (1959). Injection of C3H spleen, thymus or marrow cells in Ak new-born mice induced tolerance of C3H skin grafts in 70 to 80 per cent of the mice (Table I). The skin grafts were intact for a period of 4 to 6 months,

TABLE I.—*Tolerance of Skin Grafts in Mice Inoculated at Birth with 5 to 8 million Spleen, Thymus or Marrow Cells*

Strain of recipient mice	I.V.I. given at birth: Donor strain	I.V.I. given at birth: Cell type	Number in group	Number of mice fully tolerant
C3H/PW or C3Hf/PW	Ak_f	Spleen	143	128 (89%)*
		Thymus	161	137 (85%)*
	No cells	—	57	0 (0%)†
Ak_f	C3H/PW or C3Hf/PW	Thymus	75	51 (68%)‡
		Spleen	27	18 (67%)‡
		Marrow	32	26 (82%)‡
	No cells	—	30	0 (0%)§

* Ak skin graft intact for a total period of observation of 12 to 18 months.
† Ak skin graft rejected in 10 to 12 days.
‡ C3H skin graft intact for 4 to 6 months (see text).
§ C3H skin graft rejected in 9 to 11 days.

but thereafter the hair in some of the grafts began to fade in colour and diminish in thickness leaving a greyish-white patch. There was, however, no reaction such as is characteristically in skin grafted to non-tolerant mice. Ten Ak mice not included in Table I, inoculated at birth with C3H thymus cells, were runts and died before 5 weeks of age.

Tolerance of thymus homografts was evident from sections made of such grafts 30 or more days after grafting (Fig. 1). Such thymuses showed intact morphology. Thymus grafts in tolerant mice could often be made out as small subcutaneous nodules in or under the axilla when the overlying skin was stretched.

The development of lymphocytic leukaemia and other tumours in Ak mice tolerant of C3H

It can be seen from Table II that only one thymectomized Ak mouse grafted with C3H thymus developed leukaemia. At autopsy the lymph nodes, including the paratracheal nodes, were involved and the liver and spleen were extensively infiltrated. There was no evidence of incomplete thymectomy. The subcutaneous C3H thymus graft could not be found. On transplantation, the leukaemia grew only in Ak mice and not in non-tolerant C3H mice, suggesting that it had originated from lymphoid cells of the thymectomized Ak host.

TABLE II.—*Incidence of Mammary Tumours and Lymphomas in Ak_i Mice Inoculated at Birth with Thymus, Spleen or Marrow Cells from Adult C3H/PW or C3Hf/PW Mice**

Group†	I.V.I. cells at birth: Type	Strain	Number in group	Sex	Mice with mammary tumours: Number	Age in months	%	Mice with lymphocytic neoplasms: Number	Age in months	%
1	Thymus	C3H	16	♀	9	6–12 (average 9)	56	0	—	0
			20	♂	0	—	0	1	14	5
		C3Hf	7‡	♀	0	—	0	0	—	0
			4‡	♂	0	—	0	0	—	0
2	Spleen	C3H	5	♀	2	12, 14	40	3	7–9½ (average 8)	60
			11	♂	0	—	0	9	6½–15 (average 9)	82
3	Marrow	C3H	6	♀	2	9, 10	33	4	6–8½ (average 7)	66
			11	♂	0	—	0	9	5–10 (average 8)	82

* Total period of observation 18 months.

† Mice in all groups were thymectomized. Those in group 1 were grafted subcutaneously with C3H or C3Hf day-old thymuses. Those in groups 2 and 3 were grafted subcutaneously with isologous thymuses and received 3 further injections of spleen and marrow cells, respectively, at 4, 6 and 8 weeks of age.

‡ These mice received in addition to C3Hf thymus cells, an injection of Passage A filtrate 3 to 5 days after birth.

None of the other mice in group 1 developed leukaemia or lymphoid tumour in the thymus graft, not even those inoculated with Passage A. On the other hand, half the female mice injected at birth with normal C3H (but not C3Hf) thymus cells developed mammary carinomas (Fig. 2). Only one such tumour has been seen in over a hundred non-tolerant Ak female mice the life of which had been prolonged by thymectomy. None has ever been seen in intact Ak female mice of our colony.

Mice in groups 2 and 3 which were thymectomized and grafted with isologous thymuses developed lymphocytic leukaemia at approximately the age and frequency characteristic of the Ak strain. In most cases the subcutaneous Ak thymus graft was involved. Repeated injections of C3H spleen or marrow cells failed to alter the incidence. Some of the female mice in these two groups also developed mammary carcinomas.

Incidence of lymphoid and other tumours in C3H mice tolerant of Ak

The incidence of tumours in thymectomized C3H mice tolerant of Ak and bearing Ak or C3H thymus grafts is shown in Table III.

In group 1 (C3H mice bearing Ak thymus grafts) 14 mice developed lymphocytic neoplasms. In 10 the first sign was progressive enlargement at the site of the subcutaneous thymus graft. In the other 4, generalized lymph node involvement was evident from the onset and the graft was involved by the leukaemic process in only two. One C3H mouse, not included in Table III because it had rejected an Ak skin graft on two occasions, developed a tumour in the axilla which later proved to be a lymphoid tumour.

TABLE III.—*Incidence of Lymphomas and Other Tumours in C3H/PW or C3Hf/PW mice tolerant of Ak tissues and Bearing either Ak_i or Isologous Thymus Grafts**

Group†	Thymus donor	Recipient strain	Number	Sex	Mice with lymphomas: Number	Mice with lymphomas: Age in months	Mice with other tumours: Number	Mice with other tumours: Age in months	Mice with other tumours: Type
1	Ak_i	C3H	29	♀	6	5–16 (average 9)	9	(7–11)	M.T.
		C3H	29	♂	5	5–8 (average 5·6)	0	—	—
		C3Hf	17	♀	2	9, 10	7	(5–7)	SGT, SA
		C3Hf	22	♂	1	10	4	(6–8)	SGT, CA
		TOTAL	97		14				
2	C3H or C3Hf	C3H	6	♀	0	—	4	(7–12)	M.T.
		C3H	4	♂	0	—	0	—	—
		C3Hf	7	♀	0	—	0	—	—
		C3Hf	6	♂	0	—	0	—	—
		TOTAL	23		0				

* Total period of observation 16 to 20 months.

† Mice in group 1 were injected at birth with normal Ak spleen or thymus cells, thymectomized and grafted with a day-old Ak thymus. Mice in group 2 were treated as those in group 1 except that they were grafted with a day-old C3H thymus.

M.T. = mammary tumours.
SGT = salivary gland tumours
SA = sarcoma
CA = carcinoma
} described in text.

The histological appearance of a typical lymphoid tumour arising in a thymus graft is shown in Fig 3 and 4.

None of the mice in group 2 (tolerant C3H mice bearing C3H thymus grafts) developed lymphocytic neoplasms.

The C3H females in both groups developed mammary carcinomas characteristic of the C3H strain. Eleven mice in group 1, however, unexpectedly developed a whole array of tumours which are described below. It is significant that these eleven mice were survivors from two litters injected on the same day with the same preparation of a mixture of normal Ak spleen and thymus cells, and that they were not inoculated with Passage A or other leukaemogenic filtrate.

Immunogenetic behaviour of lymphoid tumours arising in C3H mice tolerant of Ak

The lymphoid tumours developing in tolerant C3H mice were transplanted to both Ak mice and non-tolerant C3H adult mice (Table IV). Five were transplantable to both Ak and C3H, two grew only in Ak, and five, curiously enough, only in C3H. Four of the latter had originated when their host was 10 months old or older and one at 6 months of age, after the host had spontaneously rejected the Ak skin graft.

Fate of lymphoid tumours in thymus homografts following adoptive immunization

Malignancy in the subcutaneous thymus homografts became evident with the progressive enlargement of the graft. An attempt was made to abrogate tolerance in seven mice which had developed a tumour in the graft by using lymphoid cells from non-tolerant C3H mice which were previously sensitized against normal tissues (Table V). Treatment was commenced when the grafts had reached half a centimetre in the largest diameter. The tumour completely regressed in three out of the seven treated mice. This was preceded by an intense

TABLE IV.—*Transplantation of Lymphocytic Neoplasms Arising in C3H Mice Tolerant of Ak, Thymectomized and Bearing Subcutaneous Ak Thymus Grafts*

Leukaemic donor			Results of transplantation by cells			
			In strain of origin		In Ak strain	
Number	Sex	Age in months at sacrifice	Result*	Latency† (days)	Result	Latency (days)
1	♀	12	4/6	21–40	0/6	—
2	♀	16	3/6	41–48	0/6	—
3	♂	5	0/6	—	6/6	20–30
4	♀	9	4/4	30–38	4/4	24–36
5	♀	9	3/3	34–38	2/6	36, 41
6	♀	5	0/4	—	4/4	18–30
7	♂	10	3/5	31–47	0/4	—
8	♂	8	3/3	27–31	3/3	20–25
9	♀	10	3/4	36–47	0/3	—
10	♀	7	3/5	20–31	4/4	22–29
14	♂	5	2/4	31, 33	3/4	26–32
15‡	♀	6	4/4	25–39	0/4	—

* Numerator = number of takes; denominator = number of animals.

† Interval between grafting and sacrifice or death.

‡ This mouse is not included in Table III because its tumour arose after it had rejected the Ak skin graft.

The first evidence of malignancy in mice Nos. 3, 4, 5, 6, 8, 10, 14 and 15 was enlargement at the site of the thymus graft.

TABLE V.—*Effect of Immune C3H Cells on Lymphoid Tumour Growth in C3H Mice Tolerant of Ak*

			Interval in days between		Transplantation results (Table IV)	
Number	Sex	Age at onset of malignancy (months)	diagnosis* and 1st treatment	diagnosis and death	Takes in Ak	Takes in C3H
6	♀	5	16	27	+	—
8	♂	8	1	24	+	+
10	♀	7	14	31	+	+
11	♂	5	18	†	Not done	
12	♂	5	3	†	"	
13	♀	6	1	†	"	
14	♂	5	1	27	+	+

* The day of diagnosis was arbitrarily fixed as the day when the enlargement of the graft had reached half a centimetre in its largest diameter.

† Tumour in thymus graft completely regressed from 20 to 40 days after first treatment with immune cells.

reaction in the skin graft during the second week after the first injection of immune cells. No signs of generalized leukaemia were ever present in these three mice.

The other four mice all succumbed to the disease. A feeble skin graft reaction was observed in only two and signs of dissemination of the disease became evident during the first or second week of treatment. The mice were killed when it became obvious that the treatment had failed, and the tumours were transplanted. As seen from Table IV, only one of these tumours took only in Ak. The other three were transplantable to both Ak and C3H.

The development of parotid and other tumours in C3H mice inoculated at birth with normal Ak spleen and thymus cells

The most unexpected result of the present experiments was the occurrence of parotid and sublingual salivary tumours, intramuscular sarcomas and other

tumours in eleven C3Hf mice from two litters inoculated at birth with the same suspension of normal Ak spleen and thymus cells (Fig. 5–15). Most of the mice had salivary gland tumours on both sides (either both parotids or one parotid and one sublingual gland being involved). One mouse had three primary tumours (one parotid gland tumour, one sarcoma in the pectoral muscles and one sarcoma in the thigh muscles).

All the parotid tumours examined showed essentially the same structure. Macroscopically they were made up of discrete nodules, firm, opaque, yellowish-white and of rubbery consistency. Microscopically, a thin fibrous capsule could be seen in some places only with the growth expanding and compressing it. Outside the capsule was some lymphocytic infiltration. Two main types of structure were found in the tumour proper: duct-like structures very similar to the normal ducts of the gland (Fig. 5) and a uniform population of mesenchymal-like cells with oval nuclei and delicate cytoplasmic processes. Fig. 6 shows both these patterns while Fig. 7 shows a more solid growth in which the adenomatous pattern merges into a confluent mass of cells. Under the high power (Fig. 8) many mitoses were seen in both the duct and mesenchymal cells. There were no necrotic or degenerative changes in the tumour as a whole.

Several mice had sarcomatous tumours growing in muscle tissue. These tumours were whitish-pink solid circumscribed masses without necrosis. The histological features of two such tumours are shown in Fig. 9–11. One of the tumours was in the pectoral muscles and showed much individual cellular variation (Fig. 9 and 10). Most of the cells were large with big nuclei and a greatly increased nuclear-cytoplasmic ratio. Tumour giant cells were plentiful and mitoses frequent. Cell boundaries were hard to see in some areas and a diffuse syncytium was present. Many new blood spaces could be seen and invasion of muscle tissue was evident. No cross-striation could be found in the tumour cells when the sections were stained with phosphotungstic acid haematoxylin and the tumour was best described as un undifferentiated anaplastic sarcoma. Another sarcoma in the same mouse was growing in the muscles of the thigh. The cells were mostly fusiform (Fig. 11). The nuclei were oval and only an occasional giant cell was present. The growth could be seen invading skeletal muscle and was richly supplied with new, thin-walled, blood vessels. Fine fibrils ran between individual cells and special stains showed that the tumour cells were producing a considerable amount of collagen. The tumour was undoubtedly a fibrosarcoma.

One mouse had a bilateral parotid tumour and tumours growing in both upper eyelids (Fig. 12). These latter were excised when they reached about half a centimetre in diameter and appeared to consist mostly of fibrous tissue. Histologically, they were well-differentiated growths underlying normal epidermis. They were characterized by bundles of uniform cells running in an interlacing pattern with some palisading of their nuclei although this was not well marked. Under the high power there was more nuclear variation than would be expected for a benign neurofibroma. Also many more mitotic figures were present, suggesting the histological diagnosis of fibrosarcoma of low-grade malignancy.

One mouse had a tumour growing in the abdominal cavity. It was a spherical mass with black and red patches of necrosis and haemorrhage, attached to the upper pole of the left kidney and about the same size as the kidney itself. The left adrenal could not be found. Microscopically (Fig. 13) undifferentiated cells were seen arranged in clumps and showing numerous mitoses. Invasion of blood

vessels and tumour emboli were evident in places and areas of necrosis and haemorrhage were frequent. The tumour was diagnosed as an undifferentiated anaplastic carcinoma.

No proliferative or nuclear changes were observed in the renal tubules such as has been described in mice inoculated with the polyoma virus (Stewart, Eddy and Stanton, 1959; Stanton *et al.*, 1959). There was an irregular lymphocytic infiltration in the cortex of one kidney, many small round cells being grouped mostly around small blood vessels. No inflammation was seen in the renal tubules nor was there any other evidence of pyelonephritis. The cells, themselves, appeared to be normal small lymphocytes (Fig. 14, 15) and no mitoses were found. There was no evidence of any leukaemic process anywhere in the animal.

Much of the above histological description was very kindly supplied by Dr. P. M. Sutton to whom I am very grateful.

DISCUSSION

It has been observed in the experiments reported here that high-leukaemic strain Ak mice which had been made immunologically tolerant of C3H tissues and which had been thymectomized and grafted with C3H thymus tissue did not develop lymphocytic leukaemia or lymphoid tumours in the graft. These mice are said to contain a leukaemogenic agent (Gross, 1958) and some of them received, soon after birth, an injection of Passage A filtrate, which has been shown to accelerate the onset of the disease in intact Ak mice (Miller, 1960*a*). Yet, in spite of the presence in a genetically predisposed strain of both thymus tissue (genetically foreign but tolerated) and leukaemic agent, the disease failed to develop. On the other hand, low-leukaemic thymectomized C3H mice tolerant of Ak and bearing subcutaneous grafts of normal new-born Ak thymus developed lymphocytic leukaemia or malignancy in the graft as early as 5 months after birth. The mice received no injection of leukaemogenic filtrate at birth although they were injected with normal Ak cells. However, we have failed to demonstrate that such an injection of cells, *per se*, produced leukaemia in tolerant thymectomized C3H mice bearing isologous thymus grafts (Table II) or in tolerant C3H mice with intact thymuses (Miller, 1960*a*). Clearly, therefore, the genetic susceptibility to leukaemia development must depend on an intrinsic property of the thymus tissue itself. The results obtained here are in accordance with those of Law (1952, 1957) who showed that "genetically tolerant" F_1 hybrids from crosses between high and low leukaemic strains, bearing thymus grafts from the low leukaemic parental line, did not show neoplastic change in the grafted thymus, whereas those receiving thymus grafts from the high leukaemic parental line developed a high incidence of lymphocytic neoplasms in the graft. In similar experiments, Kaplan, Hirsch and Brown (1956) showed that C57Bl, but not C3H, thymic implants developed lymphoid tumours in irradiated thymectomized (C57Bl $\times$ C3H)F_1 hosts.

There are clearly two possible ways by which the presence of an Ak thymus in a tolerant C3H host could lead to the occurrence of lymphocytic neoplasms in the host. Either (1) the Ak thymus is a source of potentially malignant Ak lymphocytes which can undergo neoplastic transformation either in their own host or in a genetically foreign but tolerant host; or (2) a non-cellular influence from the Ak thymus is responsible for the malignant change.

It would be expected on the first hypothesis that the lymphocytic neoplasms

which developed in tolerant C3H mice would be transplantable to Ak mice. Two of the early neoplasms were undoubtedly of Ak origin, growing only in Ak mice, and those that regressed following injection of sensitized lymph node and spleen cells must presumably also have been of Ak origin. The behaviour of the 5 neoplasms which grew both in Ak and C3H could be explained by reference to previous work which showed that a certain percentage of Ak leukaemias would take in C3H (Furth, Boon and Kaliss, 1944) or that, by transformation or immunoselection or both, a single passage of a tumour through a tolerant foreign host allowed subsequent growth in untreated adult mice of the foreign strain (Koprowski, Gail and Love, 1956). Finally, the five neoplasms that grew only in C3H could be spontaneous C3H neoplasms that would have developed whether the Ak thymus was present or not. At least the one arising at 16 months is likely to have been a spontaneous neoplasm. The disease is, however, rare before 12 months of age and only 2 cases have been diagnosed at 12 and 14 months in 227 untreated mice observed for a period of 14 months (Miller, 1960*a*).

The second hypothesis assumes that a malignant change takes place in a population of lymphocytes, host or donor, as a result of a non-cellular influence from the thymic epithelial reticulum cells of the Ak donor. The lymphocytic population of the compatible graft (Ak) in the genetically different but tolerant host (C3H) might undergo a change, donor-type lymphocytes being gradually replaced by host lymphocytes. On the other hand, the reticular tissue of the donor thymus might survive and induce neoplastic transformation in either donor or infiltrating host lymphocytes. This may account for the fact that most of the early neoplasms were Ak in type and the later ones C3H. Again, this situation is similar to that described by Law (1952) and Law and Potter (1956). In their experiments, AKR thymus fragments increased the incidence of leukaemia in (C3Hb × AKR)F_1 hosts, the resulting neoplasm being transplantable only to F_1 hosts. In another hybrid combination, susceptible to the leukaemogenic activity of X-irradiation, malignancy developed in thymuses from unirradiated C57Bl donors grafted to irradiated (C57Bl × A)F_1 hosts. The tumours developing early (at about 5 months) were found to be contributed by descendants of donor C57Bl thymus tissue whereas those arising later (7–10 months) were found to have originated from F_1 host cells which must have populated the graft.

The transfer of immunity against homografts of skin and transplantable tumours by lymph node cells of actively immunized mice has been described by many authors (Potter, Taylor and MacDowell, 1938; MacDowell *et al.*, 1938; Brncic, Hoecker and Gasic, 1952; Mitchison, 1953, 1954; Billingham, Brent and Medawar, 1954, 1956; Koprowski *et al.*, 1956). By using activated lymphoid cells, Medawar and Russell (1958) showed that tolerant adrenalectomized mice making use of homografts of adrenal cortical tissue could in effect be adrenalectomized, and similar results have been obtained by Krohn (1960, personal communication) with orthotopic homografts of ovaries. In three out of seven experiments described here C3H lymphoid cells sensitized against normal Ak tissues successfully caused immunologically tolerant C3H hosts to reject normal Ak skin, and Ak thymus after neoplastic transformation. One of the tumours that failed to be rejected was transplantable only to Ak mice and it is likely that the disease was too advanced when treatment was commenced. The other three tumours that could not be made to regress presumably acquired the ability to overcome any immunity of adoptive origin in the tolerant hosts for on transplantation

they grew perfectly well in both non-tolerant adult C3H hosts and in Ak mice.

The development of spontaneous leukaemia was not retarded nor was the incidence lowered in tolerant Ak mice given repeated injections of marrow or spleen cells from low-leukaemic C3H mice. A retarding effect of C3H marrow on the development of spontaneous lymphomas in (AKR × C3H)F_1 hybrids has been reported by Lorenz, Law and Congdon, (1954). It is possible that this effect is not demonstrable in the pure strain.

The occurrence of salivary and other tumours in tolerant C3H mice and of mammary tumours in tolerant Ak mice was unexpected. Only Ak females that had received C3H and not C3Hf cells developed breast tumours, which suggests transfer of the Bittner agent at birth via the cells. The C3Hf mice that developed salivary and other tumours all came from two litters injected on the same day with the same preparation of normal Ak cells ; all the surviving members of the two litters were affected. None received leukaemogenic filtrate. This particular distribution, the variety of the tumours, and the fact that the animals had all been injected on the first day of life with the same preparation of cells strongly suggests that the cells were obtained from Ak mice carrying the polyoma virus known to be present in various Ak stocks (Rowe *et al.*, 1959). Neither tissue culture (Stewart *et al.*, 1957) nor high speed centrifugation (Buffett *et al.*, 1958) is thus necessary to disclose the multipotentiality of this agent.

SUMMARY

1. Acquired tolerance of C3H and Ak thymus homografts has been achieved in Ak and C3H mice, respectively, by the intravenous injection at birth of C3H and Ak thymus or spleen cells.

2. Lymphocytic neoplasms developed in Ak thymuses grafted to thymectomized tolerant C3H mice. None were, however, seen in C3H thymuses grafted to thymectomized tolerant Ak mice.

3. The lymphocytic neoplasms arising in tolerant C3H mice were in some cases transplantable only to Ak mice, in others to both Ak and non-tolerant adult C3H, and in others only to non-tolerant C3H.

4. Three out of seven lymphoid tumours developing at the site of the Ak thymus graft in tolerant C3H mice completely regressed after treatment of the host with C3H lymphoid cells from mice previously sensitized against normal Ak tissues.

5. Other tumours occurred in these experiments. Tolerant Ak female mice developed mammary carcinomas. A group of tolerant C3H mice injected at birth with the same preparation of normal Ak cells developed multiple salivary gland tumours and other tumours.

I wish to thank Professor P. B. Medawar, F.R.S. for reading the script and for his valuable advice and criticism, and Dr. L. Brent for demonstrating the techniques of intravenous injection of new-born mice and of skin grafting. Many thanks are due to Dr. N. F. C. Gowing of the Department of Morbid Anatomy, Royal Marsden Hospital, for his help in diagnosing the tumours and to Dr. P. M. Sutton of the Department of Morbid Anatomy, University College Hospital Medical School, for his description of the histopathology of these tumours. I am indebted to Professor A. Haddow, F.R.S. and to Professor P. C. Koller for their

interest throughout this work and to the Gaggin Research Fellowship of the University of Queensland, Brisbane, Australia for its invaluable support. This investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research : Royal Cancer Hospital) from the Medical Research Council, the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

REFERENCES

Billingham, R. E. and Brent, L.—(1956) *Proc. Roy. Soc.* B, **146**, 78.—(1959) *Phil. Trans.* B. **242**, 439.

Idem, Brent, L. and Medawar, P. B.—(1953) *Nature*, **172**, 603.—(1954) *Proc. Roy. Soc.* B, **143**, 58.—(1956) *Phil. Trans.*, B, **239**, 357.

Idem and Medawar, P. B.—(1951) *J. exp. Biol.*, **28**, 385.

Brent, L.—(1959) 'Tools of Biological Research', p. 57, H. J. B. Atkins, ed. Oxford (Blackwell).

Brncic, D., Hoecker, G. and Gasic, G.—(1952) *Acta Un. int. Cancr.*, **7**, 761.

Buffett, R. F., Commerford, S. L., Furth, J. and Hunter, M. J.—(1958) *Proc. Soc. exp. Biol., N.Y.*, **99**, 401.

Furth, J., Boon, M. C. and Kaliss, N.—(1944) *Cancer Res.*, **4**, 1.

Gross, L.—(1958) *Ibid.*, **18**, 371.—(1959) *Proc. Soc. exp. Biol., N.Y.*, **100**, 325.

Kaplan, H. S.—(1950) *J. nat. Cancer Inst.*, **11**, 83.

Idem and Brown, M. B.—(1954) *Science*, **119**, 439.

Idem, Hirsch, B. B. and Brown, M. B.—(1956) *Cancer Res.*, **16**, 434.

Koprowski, H., Gail, T. and Love, R.—(1956) *Proc. Roy. Soc.* B, **146**, 37.

Krohn, P. L.—(1958) *Nature*, **181**, 1671.

Law, L. W.—(1952) *J. nat. Cancer Inst.*, **12**, 789.—(1957) *Ann. N.Y. Acad. Sci.*, **68**, 616.

Idem and Miller, J. H.—(1950*a*) *J. nat. Cancer Inst.*, **11**, 253.—(1950*b*) *Ibid.*, **11**, 425.

Idem and Potter, M.—(1956) *Proc. nat. Acad. Sci., Wash.*, **42**, 160.

Levinthal, J. D., Buffett, R. F. and Furth, J.—(1959) *Proc. Soc. exp. Biol., N.Y.* **100**, 610.

Lorenz, E., Law, L. W. and Congdon, C. C.—(1954) *Ciba Symp.*, Leukaemia Research, 184, G. E. W. Wolstenholme, ed. London (Churchill).

Macdowell, E. C., Potter, J. S., Richter, M. N., Victor, J., Bovarnick, M., Taylor, M. J., Ward, E. N., Laanes, T. and Winterstein, M. P.—(1938) *Yearb. Carneg. Instn.*, **37**, 47.

McEndy, D. P., Boon, M. C. and Furth, J.—(1944) *Cancer Res.*, **4**, 377.

Martinez, C., Smith, J. M. and Good, R. A.—(1958) *Brit. J. exp. Path.*, **39**, 574.

Medawar, P. B. and Russell, P. S.—(1958) *Immunology*, **1**, 1.

Miller, J. F. A. P.—(1959*a*) *Nature*, **183**, 1069.—(1959*b*) *Ibid*, **184**, 1809.—(1960*a*) *Brit. J. Cancer*, **14**, 83 (1960*b*) *Ibid.*, **14**, 93.

Mitchison, N. A.—(1953) *Nature*, **171**, 267.—(1954) *Proc. Roy. Soc.*, B, **142**, 72.

Potter, J. S., Taylor, M. J. and Macdowell, E. C.—(1938) *Proc. Soc. exp. Biol., N.Y.*, **37**, 655.

Rowe, W. P., Hartley, J. W., Law, L. W. and Huebner, R. J.—(1959) *J. exp. Med.*, **109**, 449.

Stanton, M. F., Stewart, S. E., Eddy, B. E. and Blackwell, R. H.—(1959) *J. nat. Cancer Inst.*, **23**, 1441.

Stewart, S. E., Eddy, B. E., Gochenour, A. M., Borgese, N. G. and Grubbs, G. E.—(1957) *Virology*, **3**, 380.

Idem, Eddy, B. E. and Stanton, M. F.—(1959) *Acta Un. int. Cancr.*, **15**, 842.

Woodruff, M. F. A. and Boswell, T.—(1954) *Brit. J. plast. Surg.*, **7**, 211.

Idem and Sparrow, M.—(1958) *Quart. J. exp. Physiol.*, **43**, 91.

THE ACTION OF FOUR CARCINOGENIC HYDROCARBONS ON THE OVARIES OF IF MICE AND THE HISTOGENESIS OF INDUCED TUMOURS*

JER K. MODY

From the Department of Experimental Pathology and Cancer Research, University of Leeds†

Received for publication March 12, 1960

THE present experiments are concerned with the state of the ovaries, including the process of tumour formation, following skin applications of four chemical carcinogens to virgin mice of the IF strain. Howell, Marchant and Orr (1954) showed that skin applications of 9 : 10-dimethyl-1 : 2-benzanthracene induced a high incidence of ovarian tumours in inbred or hydrid virgin mice of this strain as compared with three other inbred strains. Marchant (1957) also tested 20-methylcholanthrene in a small number of mice of IF origin and obtained a few microscopical tumours. In a previous paper (Mody, 1960) the normal virgin IF ovary at various ages was described and it was shown that frequent spontaneous pseudopregnancy occurs in grouped virgins.

MATERIAL AND METHODS

IF virgin females, approximately sixteen weeks old, were subjected to four fortnightly skin applications of an 0·5 per cent solution of a chemical carcinogen (obtained from L. Light & Co. Ltd., Colnbrook) in arachis oil. At each painting 1 ml. of the solution was applied as 8 drops to the dorsal and 8 drops to the ventral side of the entire trunk surface. The animals in each group were killed in batches at 0–3, 4–7, 8–11, and 12–15 weeks from the date of commencement of painting and from then onwards at 8-weekly intervals until 70 weeks. The organs were examined and the tissues fixed, cut and stained as described by Mody (1960). Between 8 and 12 weeks after the start of treatment a batch of 4 mice from each group was used for a daily three-week study of the vaginal smear.

The groups comprised :

(i) Sixty females treated with 9 : 10-dimethyl-1 : 2-benzanthracene (DMB).

(ii) Fifty-three females treated with 3 : 4-benzpyrene (BP).

(iii) Thirty-seven females treated with 20-methylcholanthrene (MC).

(iv) Sixty females treated with 1 : 2 : 5 : 6-dibenzanthracene (DB).

RESULTS

Incidence and Age of Appearance of Ovarian Tumours

Table I shows the number of tumours obtained with four chemical carcinogens, the age being counted from the date of the first painting. The tumours ranged

* Part of a thesis presented for the degree of Ph.D. of Leeds University.

† Permanent address : Indian Cancer Research Centre, Parel, Bombay 12, India.

from microscopical size to 1 cm. or more in diameter. Twelve tumours were obtained in DMB-treated mice, 4 in BP-treated and a doubtful early tumour in MC-treated, while none occurred in the DB-group. In addition, early tumours which could not be identified with certainty occurred in 4 DMB- and 4 BP-treated mice. All the tumours occurred prior to 52 weeks from the commencement of treatment.

Post-mortem Appearance of the Ovaries

At autopsy the treated ovaries were either normal, enlarged or reduced in size, the ovaries on the two sides being often of different sizes. On the whole the ovaries appeared normal to the naked eye until 33 weeks from the start of treatment and from then on showed varying degrees of enlargement culminating in haemorrhagic or non-haemorrhagic cysts or tumours ; at later ages many ovaries were small and shrunken. In the DB-treated mice, however, the ovaries showed no effect of the paintings but underwent a reduction in size with advancing age comparable to that described for untreated mice (Mody, 1960).

Cysts and tumours were always unilateral and the opposite ovaries were reduced or normal in size. Tumorous ovaries were observed with the naked eye only in DMB- and BP-treated mice. Seven DMB-treated mice had grossly detectable tumours between 16 and 51 weeks, 6 being in the right ovary. The tumours ranged from 7 mm. to 2 cm. in diameter and were fleshy pink, grey or yellowish-white in colour with darker areas of haemorrhage. The surface was smooth and slightly lobulated, the consistency soft and there were some haemorrhagic cysts. The larger tumours had loose fibrous adhesions with the peritoneal wall. In BP-treated mice two tumours were observed with the naked eye : one seen at 55 weeks was 1·5 cm. in diameter, yellowish-white in colour, with smooth surface and free from adhesions, while the other occurring at 31 weeks was only just recognisable and was greyish and slightly raised.

Histology of the Tumours

The tumours were classified as shown in Table I : granulosa cell type (12), mixed luteinised and granulosa cell type (2) and mixed thecal, luteinised and granulosa cell type (1). In addition one luteoma was induced by BP. The large tumours recognisable with the naked eye were all of granulosa cell type. In addition to the above tumours, there were very early lesions in 9 ovaries which might have been tumorous but were not certainly so.

Granulosa cell tumours

These were mainly of a compact pseudofollicular pattern. The pseudofollicles varied in size and resembled small anovular follicles. The central lumen might be indistinct or contain some eosinophilic material, the latter type resembling Call and Exner bodies. Some of the granulosa cells composing the tumours varied greatly in size and shape and showed mitosis, but most of the cells were indistinguishable from normal granulosa cells (Fig. 1). Some tumours contained papillary and cystic areas. The cysts were either large blood loculi, empty spaces or contained eosinophilic fluid. All the tumours were free from lipochrome pigment but in some there were small amounts of iron pigment in haemorrhagic areas. The microscopical tumours were usually undifferentiated.

TABLE I.—*Total Numbers, Ages of Appearance and Types of Chemically Induced Ovarian Tumours*

	I. DMB				II. BP				III. MC				IV. DB			
Incidence of tumours in total mice treated	12/60				4/53				0/37				0/60			
Survival following start of treatment	0–15	16–33	34–51	>52	0–15	16–33	34–51	>52	0–15	16–33	34–51	>52	0–15	16–33	34–51	>52
Number of mice	20	18	16	6	17	11	20	5	16	15	6	0	17	12	24	7
Granulosa cell tumours	—	5	5	—	—	1	—	1	—	—	—	—	—	—	—	—
Mixed tumours ($L + G$)	1	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—
Mixed tumour ($T + L + G$)	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—
Luteoma	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—
Doubtful early tumours	—	2	2	—	—	2	1	1	—	1	—	—	—	—	—	—

$L + G$ = Luteinised and granulosa cell types.
$T + L + G$ = Thecal, luteinised and granulosa cell types.

Mixed tumours

These were microscopical in size. In addition to granulosa cells, they contained luteinised cells (Fig. 2) and even thecal cells, the latter two cell types showing no mitoses.

Luteomas

The one tumour of this type was microscopical in size and contained a central organising haemorrhage. The component cells resembled normal lutein cells, but were more variable in size and had irregular nuclei, rarely in mitosis (Fig. 3).

One-generation transplantation of a granulosa cell tumour

The single large granulosa cell tumour induced by BP was transplanted subcutaneously into 6 mature IF male mice, in all of which palpable tumours about 2 cm. in diameter developed by 16–19 weeks. Histologically the grafted tumours resembled the original in structure. The mammary glands of the tumour-bearing males showed lobular development while there was atrophy of the seminiferous tubules and lack of secretion in the seminal vesicles. These changes were regarded as due to hormone secretion by the transplant.

A. *Processes Leading to Tumour Formation* (DMB, BP, or MC)

These may be described under the following headings (Fig. 4) :

Stage 1. Total loss of follicles.
Stage 2. Luteinisation.
Stage 3. Prominence of the germinal epithelium.
Stage 4. Occurrence of nodules.
Stage 5. Occurrence of tumours.

Total loss of follicles.—This is brought about by degeneration of all the existing follicles and failure of further formation of follicles. The earliest degenera-

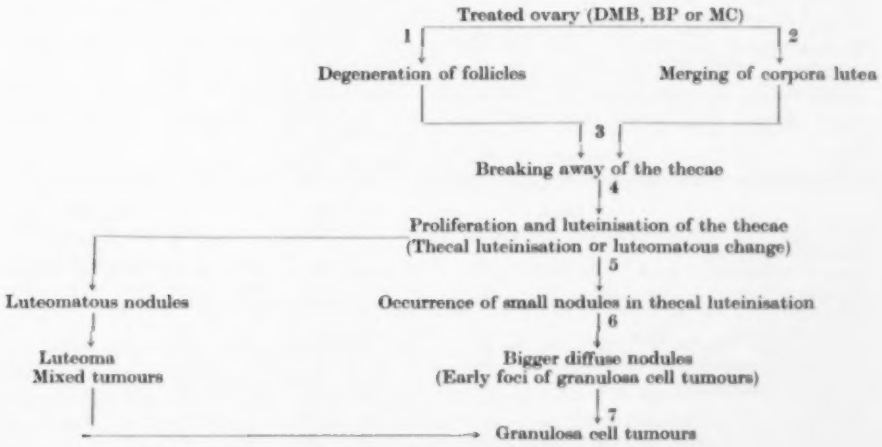


FIG. 4.—Sequence of changes leading to granulosa cell tumours.

tion was noticed in the ova, followed by changes in the granulosa cells of follicles in various stages of development (Fig. 5 and 6). These degenerative changes were not distinguishable from follicular atresia occurring in untreated ovaries (Mody, 1960). With DMB, the onset of this stage is noticed within 3 weeks following the first painting and after 16 weeks no follicles are seen. With BP and MC a few follicles might be seen until 24 weeks following the start of treatment. Atretic remnants are found at all ages.

As in untreated mice, the thecal layers of follicles undergoing atresia do not participate in the follicular degeneration. Instead the thecal cells either merge with the extra follicular tissue or aggregates of large pale dividing cells resembling thecal cells appear close to atretic follicles (Fig. 7).

Luteinisation.—At this stage two processes occur. Firstly, both young and old intact corpora lutea merge prematurely due to the breaking away of the thecae. This state may be referred to as diffuse luteinisation (Fig. 8). (It must be distinguished from the merged corpora lutea found in untreated ovaries, the lutein cells of which have completed their life span and are due to involute.) Apart from this earlier merging the process of involution follows the same course as in untreated ovaries and eventually leads to scattering of the degenerating lutein cell cords. Secondly, among the lutein cell masses of old and degenerating corpora lutea appear foci of theca-lutein or para-lutein cells, probably derived from the dividing theca-type cell clusters observed in the vicinity of atretic follicles (Fig. 7). This is a truly abnormal type of luteinisation. The newly luteinised cells are large and polygonal and nuclei are variable in size and shape, being large, round and vesicular (Fig. 9). These cells lie intermingled with the degenerated remnants of corpora lutea and are often indistinguishable from them. The intervening dark-staining spindle cells and reticulum fibres which form thin septa and characteristically ensheath the small syncytia of theca-lutein cells are helpful in distinguishing them from the corpus luteum cells. In spite of the absence of follicles and intact corpora lutea the "luteinised ovaries" are not smaller than normal ones.

In DMB-treated mice the phase of luteinisation is most prominent between 10 and 33 weeks after the start of treatment but it may start earlier or may persist to a later age (Table II). In BP-treated mice thecal luteinisation is somewhat delayed. With MC merging of corpora lutea occurs within 7 weeks and diffuse luteinisation is seen until 33 weeks, but thecal luteinisation is scanty. In all three groups the lutein cells are filled with lipochrome pigment and possess a

TABLE II.—*Microscopical Appearance of DMB-, BP- and MC-treated Ovaries*

	I. DMB				II. BP				III. MC			
Survival following start of treatment	0–15	16–33	34–51	>52	0–15	16–33	34–51	>52	0–15	16–33	34–51	>52
Number of mice	20	18	16	6	17	11	20	5	16	15	6	0
Follicle degeneration	12	—	—	—	17	2	—	—	16	1	—	—
Luteinisation	8	9	3	3	8	6	9	2	9	10	—	—
Prominence of germinal epithelium	3	4	4	2	1	4	11	4	2	8	6	—
Nodules	5	1	—	—	2	—	2	—	—	2	—	—
Atrophy	5	7	9	2	—	3	10	3	1	6	6	—
Pigment	3	4	2	2	—	2	16	5	—	7	5	—
Cystic	—	1	1	—	—	—	3	2	—	—	—	—

small pyknotic eccentric nucleus. Pigment filled phagocytes are also seen. Fibrous scars, together with hyaline degeneration in the walls of arterioles or vascular dilatation, are uncommon.

Prominence of the germinal epithelium.—Before the process of luteinisation is complete the germinal epithelium becomes high cuboidal and the ovary may become reduced in size. The prominence of the germinal epithelium is well established 16 weeks following DMB treatment and is somewhat later with BP or MC (Table II). A few dark-staining cell aggregates are seen in the stroma, but further proliferation of the germinal epithelium does not occur.

Occurrence of nodules.—Microscopical, well-defined, single or multiple nodules arising in areas of " thecal luteinisation " were found in the luteinised ovaries (Fig. 8 and 10). The component cells have bizarre nuclei with large nucleoli and the cytoplasm is less abundant than in the outer theca-lutein cells (Fig. 11). A small central lymph space is sometimes present or may appear as the nodule grows larger, at which time capillaries surround it. As nodules grow they become less well demarcated and their cells resemble granulosa cells, due to further reduction in cytoplasm. Granulosa cell tumours arise in such foci (Fig. 12), the tumour cells show mitotic activity. The tumours are always unilateral but as nodules have been observed in both ovaries it is likely that regression of nodules may occur.

Nodules were seen as early as 8 weeks following the start of DMB treatment (i.e. just after its completion) but they also arose as late as 17 weeks (Table II). Early tumour foci were found in 3 ovaries between 25 and 60 weeks. With BP a unilateral nodule was seen in the ovary in 2 mice between 12 and 15 weeks but these two nodules were not as distinct as those in the DMB group. In addition, a different variety of nodule was found in 2 mice between 34 and 51 weeks after the start of BP-treatment. This second type of nodule was luteomatous, that is composed of lutein cell cords indistinguishable from those of intact corpora lutea. The nodules occupied a considerable portion of the ovary. Also in 4 normal sized BP ovaries, at 23 to 52 weeks, there were unilateral ill-defined aggregates of granulosa cells. In two of these tiny haemorrhagic areas were seen. As these aggregates had neither ova nor thecae, they were not regarded as follicles but because of their size they were possibly very early tumours. In two MC-treated mice, a tiny nodule was seen in a normal sized but luteinising ovary on one side only at 22 and 27 weeks from the start of treatment. These nodules were of the type found in the DMB group. In one normal sized MC-treated ovary an ill-defined large granulosa cell cluster occurred at 27 weeks. It had neither ovum nor thecal layers and occupied a large portion of the ovary. As follicles are not found in MC-ovaries after 24 weeks, this might have been an early granulosa cell tumour (Fig. 13).

Occurrence of tumours.—The histology of the fully formed tumours has been described above.

B. *Processes Leading to Non-tumorous Atrophy*

All ovaries treated with DMB, BP or MC go through the stages of total loss of follicles, luteinisation and prominence of the germinal epithelium but subsequently the course of events changes in those that fail to develop nodules or from which the nodules regress. Such ovaries undergo atrophic changes, often unequal in degree on the two sides, and may finally become pinhead in size, i.e.

less than 1 mm. in diameter. The contralateral ovary of a tumour-bearing mouse undergoes atrophic changes of the same type. Qualitatively, these atrophic changes (Fig. 14) are not different from those characteristic of the ageing ovary of normal mice. The prominent germinal epithelium continues to proliferate and becomes multilayered or invaginated. The anovular buds lie in clumps near the periphery and show no activity. The dark-staining cells stream inwards from the epithelium and steadily replace the cells responsible for luteinisation (Mody, 1960). In the degenerated lutein cells lipochrome pigment is seen which may be taken up later by phagocytes. Cystic ovaries may be found occasionally.

In all the 12 mice bearing unilateral DMB-induced tumours of the granulosa cell series the contralateral ovary was atrophic. Unilateral or bilateral atrophic changes were studied in 25 DMB non-tumour-bearing mice, starting as early as 10 weeks after the beginning of treatment. In BP-treated mice all 4 contralateral ovaries of the tumour bearing mice were atrophic. In 21 of the non-tumour-bearing mice treated with BP for over 16 weeks there were atrophic changes,

EXPLANATION OF PLATES

FIG. 1.—Part of a large granulosa cell tumour of compact pseudofollicular pattern. A large cystic space containing fluid is seen towards the periphery. Thirty-five weeks after start of DMB treatment. ×90.

FIG. 2.—Part of an early mixed tumour composed of luteinised and granulosa cells. The cytoplasm in the luteinised cells is more abundant and pale staining. Fourteen weeks after the start of DMB treatment. ×60.

FIG. 3.—Part of a luteoma showing cell cords similar to the lutein cells of the corpus luteum but with a greater degree of variation in size and shape of the nuclei. Thirty weeks after start of BP treatment. ×375.

FIG. 5.—Stage of follicle degeneration. The compact dark outermost granulosa cell layer is particularly distinct in the large follicle (centre right) which is undergoing degeneration. No degenerating cells are present in the surrounding thecae. Atretic remnants and engorged capillaries are seen. Towards bottom left are corpora lutea. Three weeks after start of DMB treatment. ×90.

FIG. 6.—Earliest degenerative changes seen in the ovum. Disintegration of the nucleus and fat droplets in the cytoplasm. Three weeks after start of DMB treatment. ×340.

FIG. 7.—An aggregate of large pale theca-type cells in subgerminal position and close to an atretic follicular remnant. Some of the cells are in mitosis. Three weeks after start of DMB treatment. ×285.

FIG. 8.—Total loss of follicles. Atretic remnants (AR) and diffuse luteinisation, due to premature merging of corpora lutea, can be seen. A tiny nodule (N) towards top centre in an area of luteinisation. Eleven weeks after start of DMB treatment. ×70.

FIG. 9.—Two mitotic figures among luteinised cells, probably an area of early thecal luteinisation. Three weeks after start of DMB treatment. ×565.

FIG. 10.—A well-marked nodule towards top right in an area of luteinisation. A capillary and a lymphatic vessel are associated with it. Widespread luteinisation and scattered lymph spaces. Eleven weeks after start of DMB treatment. ×75.

FIG. 11.—A nodule surrounded by engorged capillaries in an area of luteinisation. The nuclei of the cells within the nodule are closely packed and variable in size, shape and staining. A prominent nucleolus is often present. Eleven weeks after start of DMB treatment. ×195.

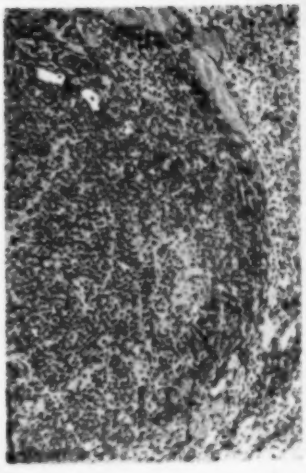
FIG. 12.—Two ill-defined nodules within an area of luteinisation. The component cells are similar to those of granulosa cell tumours. Thirty-five weeks after start of DMB treatment. ×75.

FIG. 13.—Doubtful tumour of granulosa cell type (the only one in the group), occupying a considerable portion of the ovary. Twenty-seven weeks after start of MC treatment. ×75.

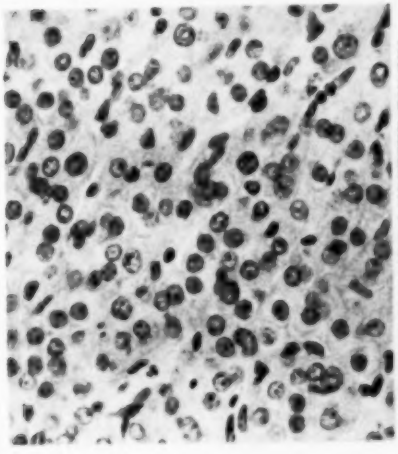
FIG. 14.—Non-tumorous atrophy. Part of a reduced ovary with prominent germinal epithelium, especially towards extreme right and clumps of anovular buds among dark-staining epithelial cells towards the periphery. Pale pigment-laden degenerated lutein cell towards centre. Fifty weeks after start of DMB treatment. ×85.

FIG. 15.—A large number of intact corpora lutea. Some of the follicles are undergoing atresia. No effect of the treatment visible. Fourteen weeks after start of DB treatment. (Compare with Fig. 7 and 9 from DMB treated mice in the same age group.) ×30.

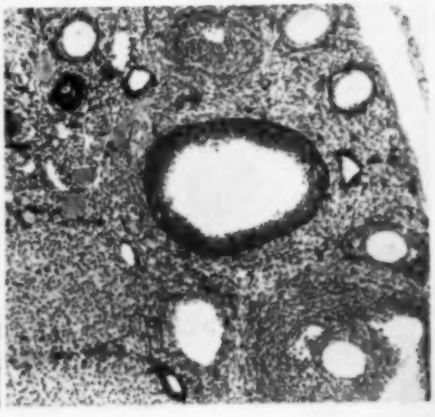
1



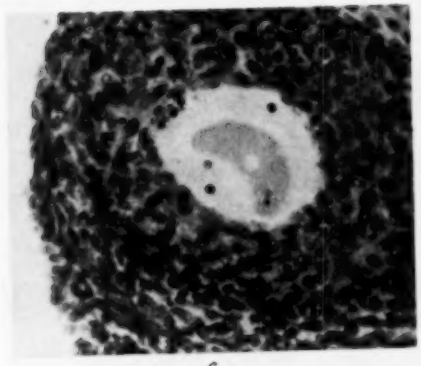
2



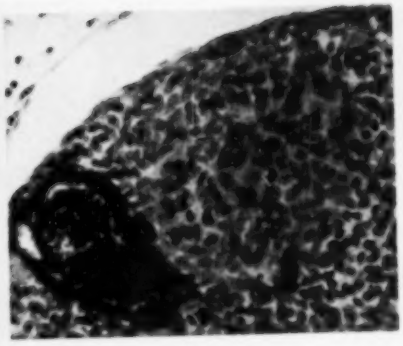
3



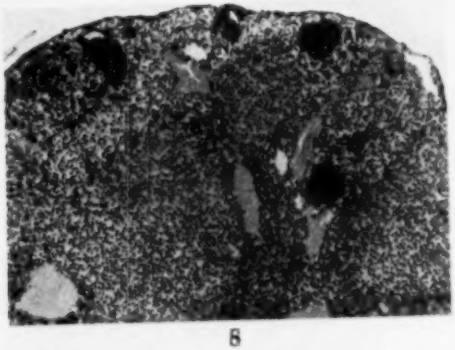
5



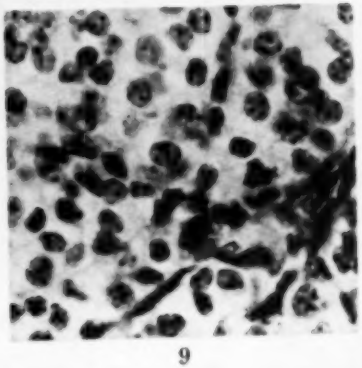
6



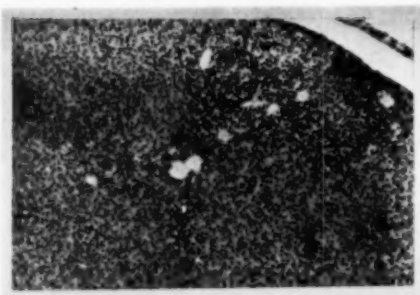
7



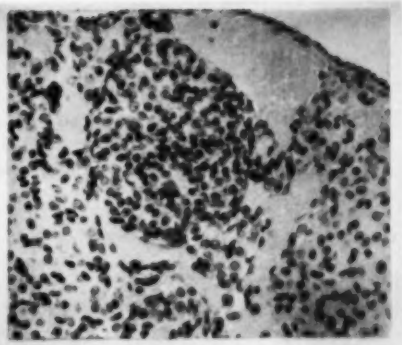
8



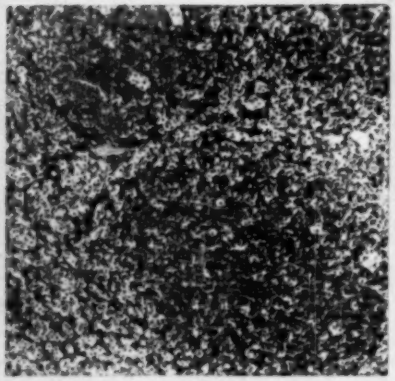
9



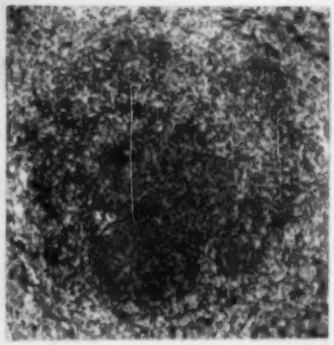
10



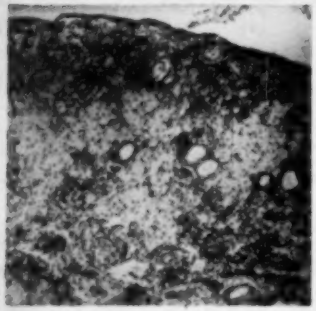
11



12



13



14

15

including 5 with cystic right ovaries. There was atrophy in one or both ovaries in 2 of the 13 mice treated with MC for 16 weeks. Small fibrous scars together with hyalinisation in the walls of arterioles and vascular dilatation were noticed in these MC-treated ovaries.

C. *Processes Leading to Senile Atrophy*

The ovaries of mice treated with DB were similar to those of normal virgin mice at comparable ages (Fig. 15), i.e. the changes characteristic of senility with only minor differences took place (Table III). Graafian follicles were somewhat

TABLE III.—*Microscopical Appearances of DB-treated Ovaries*

	Survival following start of treatment*	0–15	16–33	34–51	>52
	Number of mice	17	12	24	7
Germinal epithelium	Anovular buds	−	+	+	++
	Dark staining cells	+	+	++	++
Follicles	Primordial	+++	+	+	−
	Graafian	++	−	+	−
	Atretic follicles	+++	+	−	−
	Atretic remnants	+++	+	+	+
Corporal lutea	New	+++	+	−	−
	Old	++++	+++	+	−
	Degenerating (early)	+	+	++++	+++
	Degenerated	−	−	+	+++
	Pigment	−	+	++++	++++
	Fibrous scars	−	−	+	+

* Add 16 weeks for actual age. (Compare with Table I, Mody, 1960.)

more frequent at older ages than in normal ovaries of the same ages. Cystic follicles without haemorrhage, anovular follicles and corpora lutea atretica, i.e. atretic follicles containing lipoids (Fekete, 1946), were occasionally observed while they were rarely observed in normal ovaries. Intact young and old corpora lutea persisted until about 30 weeks after treatment i.e. until 46 weeks of age. Involution commenced within 4 weeks following the beginning of treatment and the content of degenerating (those undergoing early degeneration) and completely degenerated corpora lutea was greater than in normal ovaries, where more lipochrome pigment was present. The old DB-treated ovaries showed fibrous scarring and hyaline degeneration in the walls of small arterioles, these changes being uncommon in normal ovaries. Thus after DB treatment, total loss of follicles and diffuse and thecal luteinisation were absent (Fig. 4). Prominence of the germinal epithelium was noted with age but proliferation and invaginations were less evident than in normal ovaries. Senile atrophy was seen in 20 of the mice treated with DB, the number of pinhead or greatly reduced ovaries being larger than in normal mice.

Uterus

On microscopical examination, the state of the uterus was classed as atrophic, normal or having cystic hyperplasia. In 60 DMB-treated mice, ovarian tumours were present in 12 and cystic hyperplasia occurred in 5 of these (Table IV). By contrast, in 48 mice without ovarian tumours, cystic hyperplasia of a lesser degree occurred in 3 mice. There is thus an association between ovarian tumours and

cystic hyperplasia. When the size of the ovarian tumour was considered, it was found that the largest were often not accompanied by cystic hyperplasia. Bali

TABLE IV.—*State of the Uterus and Distribution of Breast Tumours in Ovarian Tumour-bearing and Non-tumour-bearing Mice*

		I. DMB	II. BP	III. MC	IV. DB
Ovarian tumour present	Cystic hyperplasia	1	1	–	–
	Cystic hyperplasia + breast tumour	4	–	–	–
	Normal uterus + breast tumour	5	–	–	–
	Normal uterus without breast tumour	2	3	–	–
Ovarian tumour absent	Cystic hyperplasia	2	3	2	2
	Cystic hyperplasia + breast tumour	1	–	1	2
	Normal uterus + breast tumour	5	1	5	6
	Atrophy of uterus	–	6	3	3
	Atrophy of uterus + breast tumour	–	–	6	1

and Furth (1949) made a similar observation and suggested that the oestrogenic effects come to a standstill when the tumours reach about 2 cm. in diameter.

Vaginal Smears

In view of the occurrence of spontaneous pseudopregnancy in normal IF virgins kept in fours, a 3-week study of the vaginal smear was made in small groups of treated mice 2 months after the start of treatment. Great individual variation and irregularity in the length of the cycles was noticed among DMB-, MC- and DB-treated mice. Dioestrus was long and oestrus short. By contrast, in BP-treated mice a short 6–7 day cycle occurred, with oestrus of about 2 days, and there was no mucification of the vagina at dioestrus. The cycle in BP-treated mice is thus possibly different from that of normal virgins and similar to that of anosmic mice (Mody, 1960). Further evidence is necessary to substantiate this.

Mammary Tumours

Of 60 DMB-treated mice, ovarian and breast tumours were coincident in 9 out of 12, whereas breast tumours occurred in the absence of ovarian tumours in 6 out of 48 mice (Table IV). In BP-treated mice, none of the 4 ovarian tumours was associated with a breast tumour. Twelve MC-and 9 DB-induced breast tumours occurred in the absence of ovarian tumours. Thus, although there was a high association between breast and ovarian tumours in DMB-treated mice, this association did not hold for the other 3 carcinogens. Howell *et al.* (1954) found that DMB-induced ovarian and breast tumours were dissociated, the distribution being random.

DISCUSSION

Incidence of ovarian tumours

The occurrence of 12 ovarian tumours in 60 IF mice following treatment with DMB is a lower incidence than that of 53 out of 88 previously reported by Howell

et al. (1954). In the present experiments the mice were killed at pre-determined intervals and it is to be expected that the incidence would have been higher had the animals been allowed full survival. Ovarian tumours induced by BP have not been reported previously. No unequivocal tumours were obtained with MC or DB, although pre-tumourous changes followed treatment with MC.

Sequence of ovarian changes

The first ovarian effect of DMB, BP or MC is damage to the ovum and this is followed by degeneration of all the existing follicles and failure of new follicles to develop. These effects are rather more rapid with DMB than with the other two chemicals. Abnormalities of luteinisation are followed by the appearance of nodules (Fig. 9 and 10). These are derived from theca-lutein cells and are thought to be the starting point of the tumours of the granulosa cell series. These nodules may be bilateral, although the tumours are always unilateral, and it is therefore suggested that some regress. The final stage in the development of the nodules into tumours was seen after DMB and BP treatment but was not reached after MC treatment in these experiments. From histological examination it is not possible to be certain when the growth of the nodules becomes autonomous. If nodules fail to develop, or regress, the ovaries undergo regressive changes characterised chiefly by proliferation of the cells of the germinal epithelium, which stream inwards to replace the lutein tissue.

By contrast, the changes observed after DB treatment resemble those seen in ageing normal virgins. Total loss of follicles did not occur, abnormal types of luteinisation and nodules were absent and there were no tumours. The specificity of action on the ovary of these chemicals, all of which are carcinogenic to other organs (e.g. the skin), is thus apparent.

The histogenesis of the tumours induced by DMB and BP is similar to that seen in irradiated ovaries (Brambell and Parkes, 1927; Giest, Gaines and Pollack, 1939) and in intrasplenic ovarian grafts (Biskind and Biskind, 1949). The sequence of events after chemicals may include a transitional luteomatous stage but this phase seems to be less persistent than that observed by Lipschutz (1960) and Lipschutz, Rojas, Cerisola and Iglesias (1960) in intrasplenic or fragmented ovaries. From the present experiments it appears that granulosa cell tumours can arise from areas of abnormal luteinisation, without an intervening luteomatous phase, but this is not certain.

Relation between the occurrence of ovarian and breast tumours

Following DMB, ovarian and breast tumours frequently occurred in the same mouse but there was no such association when the carcinogen was BP, MC or DB. Marchant (1959) made the interesting observation that ovarian tumours did not develop in normal mouse ovaries grafted into DMB-treated mice, although breast tumours did occur, and that the ovaries of treated mice did develop ovarian tumours when grafted into normal mice.

Hormonal effects of the ovarian tumours

Of the 12 mice bearing DMB-induced tumours, there were 5 in which cystic hyperplasia of the uterus was observed (Table IV), and it was also present in the one mouse with a large BP-induced tumour. However, it is an inconstant feature

and can only be regarded as a crude index of secretory activity, depending on the ratio of oestrogen to progesterone rather than upon actual quantities. When the BP-induced tumour was grafted into male mice evidence of a feminising effect was seen in lobular development in the breast, suppression of spermatogenesis and lack of secretion in the seminal vesicles.

SUMMARY

Limited skin applications of four carcinogens (DMB, BP, MC and DB) were made to inbred virgin IF mice, which were subsequently killed at ages ranging from 16 to 70 weeks in order that the sequence of ovarian changes might be studied.

Ovarian tumours of the granulosa-cell series were induced by means of DMB and BP, but not with MC or DB. The induction period was shorter with DMB. Pre-tumorous changes were induced with MC, but DB exerted no effect upon the ovary.

The tumours were unilateral and of the granulosa cell series, the granulosa cell type being predominant. They resembled those occurring spontaneously in some strains of mice, those induced by X-irradiation or in intrasplenic ovariar grafts in castrates and the granulosa cell tumours of the human ovary.

The sequence of histological changes in the ovary after treatment with DMB, BP or MC is death of the ova and degeneration of all the follicles, failure of new follicles to develop, merging of the corpora lutea, proliferation and luteinisation of theca cells and formation of multifocal nodules from these luteinised theca cells in one or both ovaries. The tumours arise unilaterally in one or more nodules, the remainder of which undergo regression. The secondary proliferation and luteinisation of the theca cells following merging of the corpora lutea, with subsequent nodule formation, is regarded as the essential precursor of tumour formation. The ovary contralateral to the tumour-bearing ovary, or both ovaries where no tumour is present, undergoes reduction in size. This is characterised by a streaming into the substance of the ovary of dark staining cells derived from the germinal epithelium and the accumulation of lipochrome pigment in phagocytes.

Following treatment with DB the normal age changes which take place in virgins occur (Mody, 1960). Large cystic follicles, persistent corpora lutea, fibrous scars and hyaline degeneration of blood vessel walls are more frequent than in the normal and some ovaries become greatly reduced in size.

REFERENCES

Bali, T. and Furth, J.—(1949) *Cancer Res.*, **9**, 449.
Biskind, G. R. and Biskind, M. S.—(1949) *Amer. J. clin. Path.*, **19**, 501.
Brambell, F. W. and Parkes, A. S.—(1927) *Proc. Roy. Soc.* B, **101**, 316.
Fekete, E.—(1946) *Cancer Res.*, **6**, 263.
Giest, S. H., Gaines, J. A. and Pollack, A. D.—(1939) *Amer. J. Obstet. Gynec.*, **38**, 786.
Howell, J. S., Marchant, J. and Orr, J. W.—(1954) *Brit. J. Cancer*, **8**, 635.
Lipschutz, A.—(1960) *Acta. Un. int. Cancr.*, **16**, 149.
Idem, Rojas, G., Cerisola, H. and Iglesias, R. (1960) *Ibid.*, **16**, 206.
Marchant, J.—(1957) *Brit. J. Cancer*, **11**, 452.—(1959) *Ibid.*, **13**, 306.
Mody, J.—(1960) Thesis presented for the degree of Ph.D. of Leeds University.

MAMMARY TUMOUR INCIDENCE IN RELATION TO AGE AND NUMBER OF LITTERS IN C_3H_f AND $RIII_f$ MICE

B. D. PULLINGER AND S. IVERSEN

From the Cancer Research Department, Royal Beatson Memorial Hospital, Glasgow, C.3.

Received for publication February 16, 1960

REFERENCE data in respect of age, number of litters and mammary carcinoma incidence in C_3H_f/He and $RIII_f$/Pu mice have been recorded and analysed quantitatively for the purpose of eventual comparison with results from substituted ovarian hormones. The incidence of tumours of other sites is included.

MATERIALS AND METHODS

Origin of mouse strains

Some particulars of the first 34 generations after cross-suckling 2 females and 1 male of an RIII litter comprising 482 former breeding females have previously been reported (Pullinger, 1952*a*, 1955). Absence of evidence of mammary tumour agent from extracts of 2 tumours derived from the cross-suckled strain and tested in susceptible agent-free F.1 hybrids of C57 mothers and RIIIb fathers, together with an overall reduction in mammary carcinoma from 80 to less than 3 per cent in breeders and from 69 per cent to nil in virgin females through 34 generations allowed the presumption that the agent had been excluded from all sublines. The present report concerns generations 35 to 52 since cross-suckling. The number of litters a female was allowed to bear was deliberately limited to 3 in the majority of breeders in F.40 and to 6 in F.41 to F.44 but in all others breeding was unrestricted (Table I) and was interrupted only for the purposes of securing the next generation or sufficient animals for experiment. Twenty-four breeders only were self-limited to one or two litters.

Progeny of C_3H_f mice were derived from a litter in the F.23 generation which was given to this hospital in 1954 by Dr. W. E. Heston. This substrain was derived by Caesarian section and cross-suckling from Andervont's C_3H line (Andervont and McEleney, 1941) in which mammary tumour incidence was higher in virgin females than in breeders. Progeny of the cross-suckled C_3H_f/He substrains were exhaustively tested for evidence of agent and none was found by Heston and his colleagues (Heston *et al.*, 1950; Heston and Deringer, 1952; Heston, 1953; Heston, Deringer and Dunn, 1956; Heston, 1958). Breeding in these laboratories has been carried out by brother and sister matings supervised and recorded by one of the authors. With the exception of 17 out of 108 females breeding was unlimited and was interrupted by removal of pregnant females from breeding boxes only for the purpose of rearing litters as required. After weaning the mothers were returned to their breeding boxes.

Breeding females of both strains were examined daily. Those that had ceased breeding were examined for tumours once weekly.

TABLE I.—*Tumour Incidence, Average Survival Age and Number of Litters of 544 RIII, Breeding Females Living to 8 Months or More*

Filial generation	Number of sub-lines	Number of breeders	Mean: Number of litters	Mean: Age at death† (in months)	Number of females with: Mammary carcinoma	Osteo-sarcoma	Lung adenoma	Lympho-blastoma	Miscellaneous tumours
35	14	67	8·16	18·4	6 (17·5)	1 (10·5)	3 (22·9)	15	2 Epitheliomas of clitoris and nipple (22·0). 1 Subcutaneous sarcoma (21·0). 2 Hepatomas in siblings (23·5). 1 Sarcoma of liver (19·2).
36	12	39	8·53	19·1	1 (21·6)	1 (17·1)	1 (26·5)	13	1 Atypical skin tumour (24·0).
37	15	41	8·78	20·5	3 (13·1)	0	1 (21·9)	20	—
38	14	36	7·11	17·2	0	0	1 (22·2)	11	1 Squamous carcinoma of anus (17·2). 1 Columnar carcinoma of rectum (20·5). 1 Squamous carcinoma of clitoral gland (11·7).
39	22	48	7·96	17·4	0	2* (8·5)	0	14	1 Parotid tumour (21·4). 1 Uterine fibroid (21·8).
40	20	62	3·21	21·0	0	0	4 (21·1)	26	Bilateral sarcoma of ovaries (11).
41	18	37	5·43	19·6	0	0	0	18	Intramandibular carcinoma (27·7).
42	11	32	6·09	18·5	0	0	1 (26·2)	13	1 Hepatoma (20·8).
43	6	17	5·65	17·6	1 (11)	0	1 (22·5)	4	1 Fibrosarcoma of uterus (17·9).
44	9	16	6·50	18·0	0	1 (7)*	0	11	1 Sarcoma of rectum (14·1).
45	6	19	7·74	18·1	0	1 (10)	1 (21·2)	7	—
46	10	28	7·79	19·6	1 (15·9)	0	0	11	—
47	10	23	7·74	21·5	0	1 (13)	1 (20·1)	6	—
48	6	17	5·59	20·8	0	0	1 (21·5)	4	1 Fibroma of uterus (24).
49	4	12	8·33	17·9	2 (17·2)	0	0	0	1 Intramandibular carcinoma (26·6).
50	2	10	7·80	22·9	0	0	0	1	1 Hepatoma (20·3).
51	5	21	7·95	20·4	0	3 (16·2)	0	4	—
52	7	19	8·32	22·9	0	0	0	10	1 Adenocarcinoma of stomach (25·7).
Totals 18		544			14	10	16	188	

The common ancestor belonged to F24.
Age or average age of tumours in brackets.
* The F39 and one in F44 not included in population at risk.
† Or mammary tumour found.

Both strains were housed in the same room in zinc or galvanised iron cages with wire mesh lids and sawdust and wood shavings. Food in the form of cubes of composition 41 (of the Medical Research Council's Laboratory Animals Centre) and drinking water were supplied *ad libitum*. Every three months for a period of 3 weeks streptomycin, 0·025 per cent, was added to the drinking water to avoid epidemics of Tyzzer's disease. Six months after arrival of the C_3H_f litter the room was air-conditioned with an electrostatic precipitator to reduce atmospheric pollution for other purposes and was kept at 78–80° F. All animals were examined weekly for tumours or other disease. They were allowed to live out their lives and were killed only when moribund or unable to feed or drink or when a tumour had developed. Tissues for microscopic examination and for bulk-staining were fixed as a routine in Bouin's fluid or in other fixatives as stated. A few of the more dense mammary adenomas (hyperplastic nodules) were examined microscopically. In this way some presumed early carcinomas were detected but because all such nodules were not examined, none has been included among the gross, palpable tumours upon which incidence is customarily based. Grafts of some tumours were made in males and females of their respective strains or in F.1 hybrids. Biopsies were done on a sample of tumour bearers. The incidence of other tumours is recorded with the exception of the lymphoblastoma group in C_3H_f.

RESULTS

RIII$_f$

The overall incidence amounted to 14 mammary carcinomas in 544 breeders in 18 generations (Table I). The 14 $RIII_f$ mammary tumours were less readily typed according to the description by Dunn (1959) than were those in C_3H_f mice. Type A, of uniform fine acinar structure, and Type B, a group of diverse acinar, cystic and papillary formations, merged more often. As observed by Foulds (1956) compound organoid carcinoma was relatively common. With these reservations there were 5 malignant adenoacanthomas (1 organoid), 2 anaplastic carcinomas, 4 type B tumours and 3 compound organoid carcinomas without squamous change. Of the total $RIII_f$ mammary tumours seen since cross-suckling, amongst 1026 breeders 21 were in anterior and 5 in posterior nipple areas, a distribution consistent with that of $RIII_f$ adenomas (Pullinger, 1952b) and with mammary carcinoma in C_3H_f females (Prehn, Main and Schneiderman, 1954). The latter authors found also that the degree of unevenness of distribution was largely a function of tumour age. The greater the tumour age the greater was the percentage of anteriorly occurring tumours. In a very much smaller number of $RIII_f$ and C_3H_f mammary tumours seen by the present authors this relationship does not appear to hold good (Table II).

The incidence of the group of tumours including lymphoblastoma, reticulum-celled neoplasias and leukaemia rose in successive generations; in F.44 it was 68 per cent, whereas in F.1 to 14 there were 15 examples in 100 breeders. The change in anatomical distribution was as striking as the increase in incidence. In early generations regional and abdominal lymph nodes were mainly affected; only 26 per cent of lesions occurred in liver or spleen, whereas in F.40, for example, 88 per cent of all these tumours affected the latter organs, the liver predominating. Two main types of these liver lesions were seen, a neoplastic extramedullary

TABLE II.—*Distribution of Spontaneous Mammary Carcinoma According to Age, Strain and Site*

Strain	Nipple areas	Age in months 11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	Totals
$RIII_f$	Anterior	2	0	2	4	1	3	0	0	0	2	2	2	2	0	1	0	0	0	0	0	21
	Posterior	0	0	0	0	0	1	0	1	1	1	0	0	1	0	0	0	0	0	0	0	5
C_3H_f	Anterior	0	1	0	0	1	0	1	3	0	1	2	0	0	3	2	1	2	2	1	2	22
	Posterior	0	0	0	0	0	0	0	0	0	0	0	2	2	0	0	0	0	0	2	0	6

erythropoesis and polymorphic reticulum-celled growths. This large increase in a more lethal type of tumour than are lymph node lesions might have reduced the average survival age to below that at which mammary carcinoma would arise but this was not so. The average age at death of the first 482 breeders in F.1–34 was 20 months and the average tumour age was 19 months. In F.35 to 52 generations comprising 544 breeders the average tumour age was 19·6 months, and survival age 19·4 months.

Fewer tumours of other sites were observed and usually at a later age with the exception of some sarcomas (Table I). The earliest of these, two osteogenic sarcomas of bone, were found in two 7 month old mice. Bone tumours were found slightly less often than mammary carcinoma and occurred at random, only occasionally showing familial relationships. Three which arose in F.51 were all descended from a common grandmother in F.49. This female developed a mandibular carcinoma at 27 months of age, containing hair shafts similar to tumours described by van Rijssel and Mühlbock (1955). In F.39 a brother and sister had osteogenic sarcomas, the female in the right femur, the male in the right foreleg above the paw. The common ancestor, without this tumour, of all in Table I with osteogenic sarcoma, belonged to F.32. No other near relationships were seen. Though relatively few males were kept to old age the predominance of bone tumours in females noted in Simpson mice by Pybus and Miller (1940) was less striking in the $RIII_f$ strain. One osteogenic sarcoma in 100 $RIII_f$ male breeders of the same generations was observed but others were found in males set aside for experiments unconnected with induction of tumours.

Hepatoma was uncommon and none was seen before F.25 although these growths had been sought. The usual preponderance in males over breeding females was found but not over virgin females which had a similar incidence (Table III). These results are referred to again with findings in the C_3H_f strain. Intracytoplasmic inclusions which have been described by Head and Laird (1956) were found in all except one $RIII_f$ hepatoma. An unusual group of growths occurred in the rectum in some breeders and virgin females. These were either carcinomas of rectal mucosa, sarcomas or mixed tumours invading the musculature. Some were associated with cystic epithelium lying between longitudinal and circular muscle fibres. The same relationships may have been present in others but serial sections were not made. One parotid tumour in a breeder in F.39, 1 among the progeny of females mated at random for production of experimental animals, and 1 in an ovariectomised breeder have been seen but none before that generation. An epithelial tumour of subcutaneous tissue probably derived from epidermis was not classified.

C_3H_f

One hundred and eight females were bred in 11 generations. Breeding of 17 out of 108 was limited to 10 to 11 months of age for the purpose of inclusion in a reference group to be recorded at another time. Four of these 17 developed mammary carcinoma and 24 of the remaining 91. In the present analysis all have been considered together as one group of 108 breeders comprising the population at risk. Twenty-two carcinomas occurred in the 3 anterior pairs of nipple areas and 6 in the 2 posterior pairs, a proportion higher than, but corresponding with that found by Prehn, Main and Schneiderman (1954) (Table II). Of Type A (uniformly acinar) there were 11 examples, of Type B (multiform acinar, cystic and papillary) there were 12, and of Type C, composed of small uniform epithelial-lined cysts enclosed in layers of spindle cells, there were 2. One malignant adenoacanthoma and 2 carcinosarcomas were diagnosed.

None of the 28 mammary tumours was associated with pituitary enlargement or adrenal cortical carcinoma. Two were associated with small ovarian granulosa-celled tumours. Forty-six pairs of adrenal glands were examined microscopically. Proliferation of subcapsular A cells, usually fusiform with deeply stained nuclei and scanty cytoplasm, and the change from lipoid to compact fasciculata cells had occurred in all. Large rounded or polygonal vacuolated pale staining B cells were found in clusters in the cortex of one or both of 18 pairs of adrenals and ceroid (chromolipoid) in 11 pairs mainly in older animals. Cortical B cells were found in 10 of 22 breeders with mammary carcinoma in 4 of which they were hyperplastic ; they were found in 8 out of 24 without carcinoma and in 2 were hyperplastic. Alphabetical typing of abnormal adrenal cells is in accordance with the description of Woolley and Little (1945). The compact fasciculata cells resembled those previously described in virgin C_3H_f females (Pullinger, 1959) which are found also in males. Three microscopic medullary adenomas, one extracapsular adenoma of compact cells only and one of both A and compact cells were found. These extracapsular nodules of compact cells can now be identified as accessory adrenals which have undergone the same age changes as the adrenal glands. Accessory mouse adrenals (described by Whitehead, 1932) have now been found in C_3H mice by Hummel (1958). Adrenal glands and nipple areas of the same 18 females with, and of 16 without, mammary carcinoma were examined microscopically for correlations between the presence of B cortical cells and failure of involution or of hyperplasia of mammary glands. No correlations were found. Of 7 hepatomas 4 were associated with mammary carcinoma in breeding females.

The incidences of other kinds of tumours were as follows : 5 ovarian granulosa-celled or tubular adenomas often accompanied by cysts and 1 ovarian fibroma ; 8 lung adenomas ; 3 sarcomas of soft tissues ; 1 carcinoma of a uterine horn ; 1 wart of skin, and 1 sarcoma of an occipital bone. Multiple bone forming foci were found in the lungs of one animal with no post mortem or other record of a primary growth elsewhere. This animal had a small mammary tumour and the white nodules seen at necropsy in the lungs were thought to be metastases but were bony structures. Mesenteric disease of lymph nodes characteristic for the strain (Simonds, 1925 ; Dunn, 1953) was common.

Incidences of hepatoma in males and breeding females of both $RIII_f$ and C_3H_f strains are in accord with most previous observations reviewed by Andervont (1950) and added to by Agnew and Gardner (1952). The figures in Table III

show a predominance in males and non-breeding females over breeding females in both strains and support the suggestion of Burns and Shenken (1943) that incidence in virgin females is nearer to that in males.

TABLE III.—*Incidence of Hepatoma*

Strain, sex and parity of mice	Mice alive at 16 months and over	Number with hepatoma	Percentage with hepatoma
C_3H_f Females :			
Breeders	103	7	6·8
Virgins	110	27	24·5
Males	57 at 15 months and over	17	29·8
$RIII_f$ Females :			
Breeders	419	4	0·93
Virgins	32	3	9·4
Males	90	8	8·8

No intracytoplasmic inclusions have been found in any of the C_3H_f hepatomas. Hepatoma and hepatic reticulum-celled tumours were found together in 2 $RIII_f$ animals. Without microscopic examination the liver-celled growth might have been missed. The deeply groved channels in which their surface blood vessels lie draw attention to the presence of hepatomas either alone or when combined with lymphoblastoma.

Second primary mammary carcinomas and grafts

The simultaneous appearance of more than one primary mammary tumour when associated with milk factor is common. Several were reported by Heston *et al.* (1950) in C_3H_f females. None was seen among 28 tumour bearers here recorded but in 4 out of 10 of the latter which lived the same length of time or less than the remaining 6, a second primary mammary carcinoma was found at 49, 67, 74 and 79 days after excision of the original primary. The appearance or non-appearance of a second tumour was unrelated to the number of hyperplastic (adenomatous) nodules in mammary glands. No nodules were found in one mouse with a second tumour and the average numbers in the 2 groups were similar. Recurrences of primary growths occurred in 8 out of 10, pulmonary metastases in 2 of the 8.

First generation grafts of mammary carcinoma were made into C_3H_f or F.1 hybrid mice, 4 into males only, 13 into males and females and 1 into females only. By chance the last was a C tumour, a type which rarely takes. Grafts which reproduced the distinctive morphology of the latter tumour grew and were palpable in 3 months in all 4 tests females. Fifteen of the 17 grafts made in males grew in all grafted animals. In one of the 17, one graft out of 3 had not grown in 2 months when the mice were killed. All of 3 grafts from another tumour failed to grow in males in 7 months but the grafted sites were found at necropsy. Sections revealed apparently viable adenocarcinoma cells and tubules in dense collagen in all three. Of 14 tumours grafted into females, one failed to grow in 2 of 3 hosts in 2 months but apparently viable cells were found in sections of the grafted site. The latent period between grafting and growth of first generation transplants of different C_3H_f mammary carcinomas and of different fragments of

the same tumour varied considerably. There was no C_3H_f tumour which failed to grow in every grafted host but the same irregularity in " takes " encountered by Andervont and Dunn (1952) in transplanting hepatomas in this strain was met with among mammary adenocarcinomas in spite of homozygous histocompatibility. Growth of $RIII_f$ grafts was always successful in $RIII_f$ males and the rate of growth usually more rapid and uniform.

Analysis of breeding records and mammary carcinoma incidence

In Fig. 1 the probits of the incidence of mammary tumours are plotted against the number of litters. The incidence is calculated as the number of tumours amongst the difference in number of females having had n and $n + 1$ litters. Thus $F = N^*/N_d$, where F is the incidence, N^* the number of animals with tumours and N_d the difference in number of females having had n and $n + 1$ litters.

A are Jones' (1940) data analysed by Shimkin (1945) for mice of the A strain possessing the Bittner agent ; B are Heston's (1958) data for C_3H_f mice without Bittner agent ; C the data for C_3H_f agent-free mice given in Table IV) and D are the data for $RIII_f$ agent-free mice given in Table V.

TABLE IV.—*C_3H_f Mice*

Number of litters ($= n$)	Number of females ($= N_d$)	Number with tumours ($= N^*$)	Incidence of tumours $\left(F = \frac{100N^*}{N_d}\right)$	Average age of N_d (days)	Time of tumour appearance (days)
1	9	1	11·1	252	784
2	14	1	7·1	221	907
3	11	3	27·3	258	616, 730, 858
4	19	3	15·8	314	586, 845, 903
5	20	4	20·0	322	696, 732, 750, 868
6	10	4	40·0	343	530, 688, 807, 842
7	6	2	33·3	367	514, 730
8	9	6	66·7	428	372, 452, 533, 754, 803, 856
9	0	0	0	360	—
10	5	1	20·0	377	529
11	2	2	100·0	499	618, 670
12	3	1	33·3	443	670
	108	28			

As can be seen from this figure all the data conform to a straight line course which suggests, as pointed out by Shimkin (1945) that " the effect of pregnancy upon mammary carcinogenesis (in strain A) is logarithmic in its accruance ". It is thus the number of pregnancies a mouse has had which is thought to increase the tumour risk, a point of view which also has been expressed by Mühlbock (1950) and by Heston (1958). Fig. 1 shows undoubtedly that the probits of N^*/N_d increase with increasing litter number, but it is noteworthy that the straight lines in B, C and D, where the lines marked a represent the regression lines for the probit and those marked b the regression lines for the working probits, have practically identical slopes. This would indicate that the increase in tumour risk was constant and independent of the strain of mice, which is contrary to

observed findings. It seems therefore likely that the linear course obtained when the data are calculated and plotted as in Fig. 1 gives a sort of relative measurement and is only indicative of a possible identical mechanism of carcinogenesis which is independent of the strain and of the presence of the Bittner agent. In Fig. 2 the probit of the incidence (N^*/N_d) is plotted against the average age of the N_d

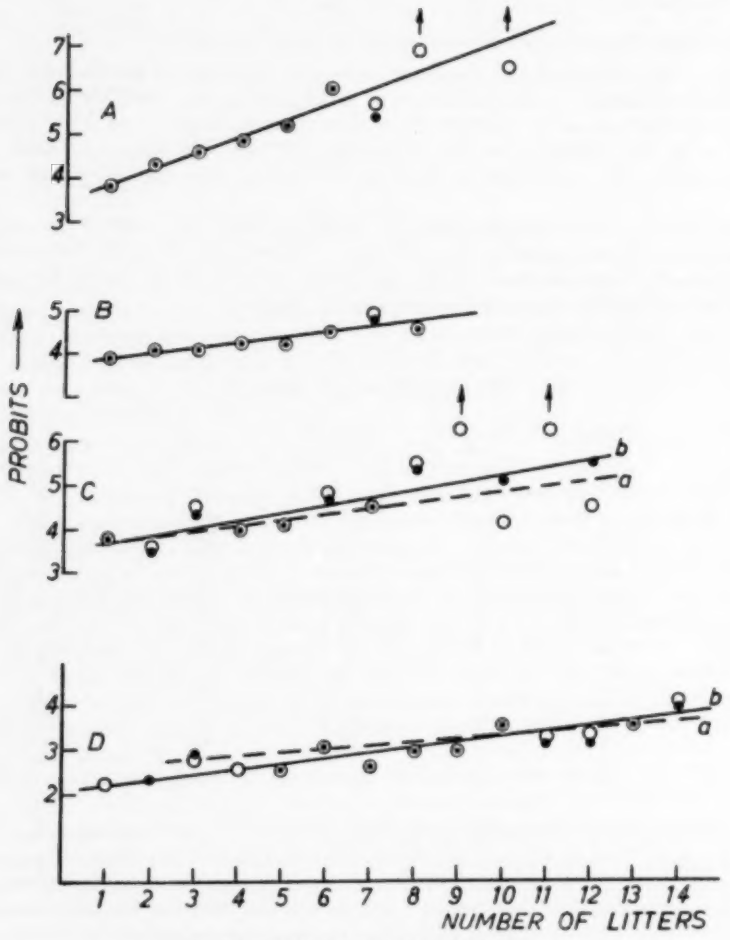


FIG. 1.—Ordinate: Probits of incidence.
A: Strain A (Jones, 1940).
B: C_3Hf (Heston, 1958).
C: C_3Hf, Table IV.
D: RIIIf, Table V.
Abscissa: Number of litters.
● = Working probits.
○ = Probits.

TABLE V.—*RIII$_f$ Mice*

Number of litters ($= n$)	Number of females ($= N_d$)	Number with tumours ($= N^*$)	Incidence of tumour $\left(F = \frac{100N^*}{N_d}\right)$	Average age of N_d (days)	Time of tumour appearance (days)
1	6	0	—	110	—
2	18	0	—	147	—
3	63	1	1·59	156	701
4	45	0	—	206	—
5	55	0	—	236	—
6	104	3	2·88	256	330, 404, 436
7	47	0	—	277	—
8	43	1	2·33	302	327
9	43	1	2·33	348	623
10	24	2	8·33	392	485, 490
11	27	1	3·70	386	563
12	27	1	3·70	444	425
13	12	1	8·33	459	497
14	17	3	16·64	463	417, 477, 648
15	8	0	—	460	—
16	4	0	—	494	—
17	1	0	—	526	—
	544				

mice and, as can be seen, the points are distributed randomly around straight lines, which now have significantly different slopes. It would therefore be even more justifiable to suggest that the effect of age upon mammary carcinogenesis

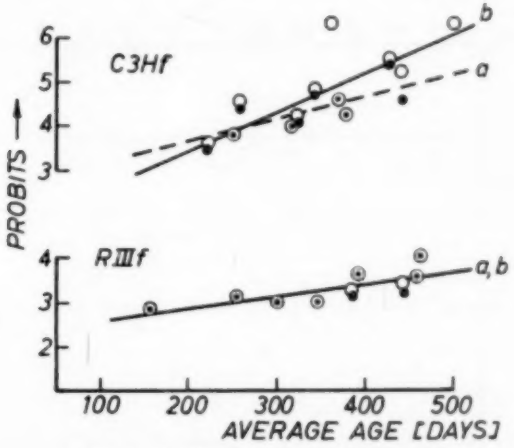


FIG. 2.—Ordinate: Probit of incidence.
Abscissa: Average age in days.
● = Working probits.
○ = Probits.

is logarithmic in its accruance. Fig. 3, which shows the survival curves, illustrates the well known finding that breeders are less long-lived than non-breeders. But from Fig. 4 and 5 where the average age at death for the N_d mice is plotted against

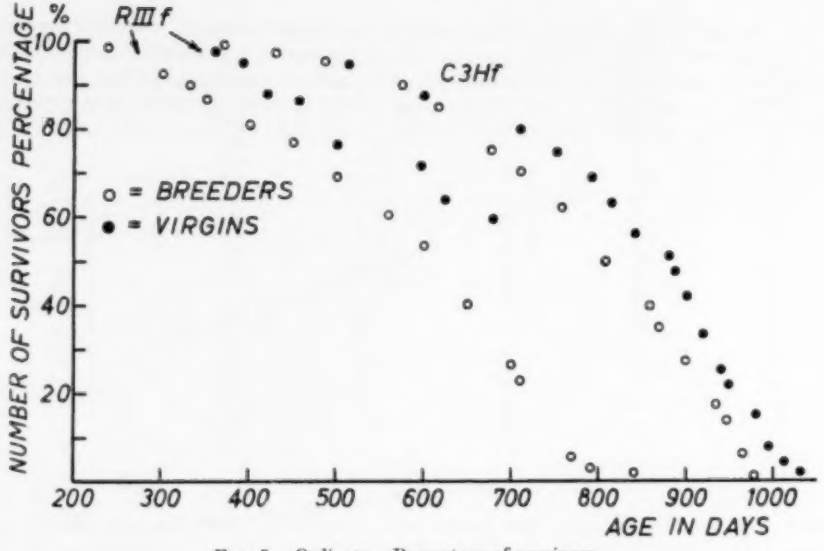


FIG. 3.—Ordinate : Percentage of survivors.
Abscissa : Age in days.

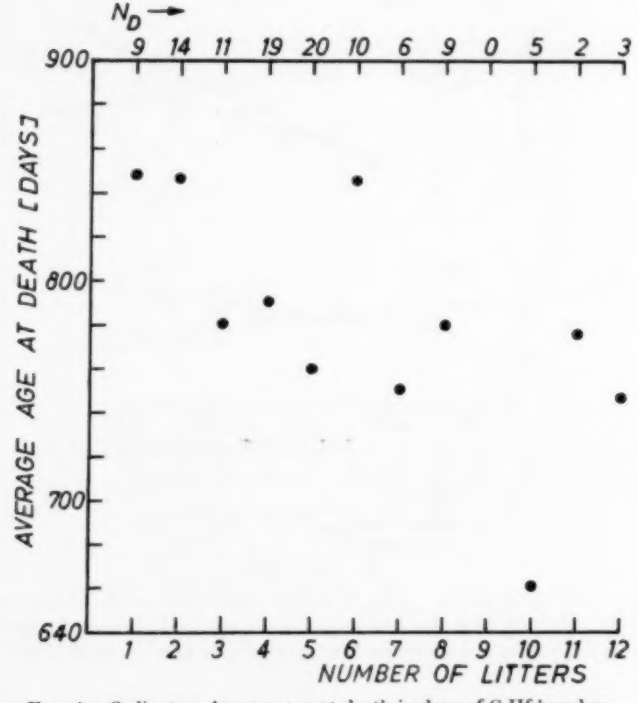


FIG. 4.—Ordinate : Average age at death in days of C_3Hf breeders.
Abscissa : Number of litters.

the litter number for the C_3H_f and $RIII_f$ strains, respectively, it can be seen that the points in the two figures are distributed in diametrically opposite directions. In Fig. 4 they follow a downward trend indicating a decrease in death age with increasing litter number, while in Fig. 5 the death age if anything increases with increasing litter number. As the probits of the incidence give a straight line course when plotted against the number of litters and when plotted against age the inference that can be drawn is that the probit procedure indicates the existence of an identical basic mechanism, but does not permit conclusions as to the effect

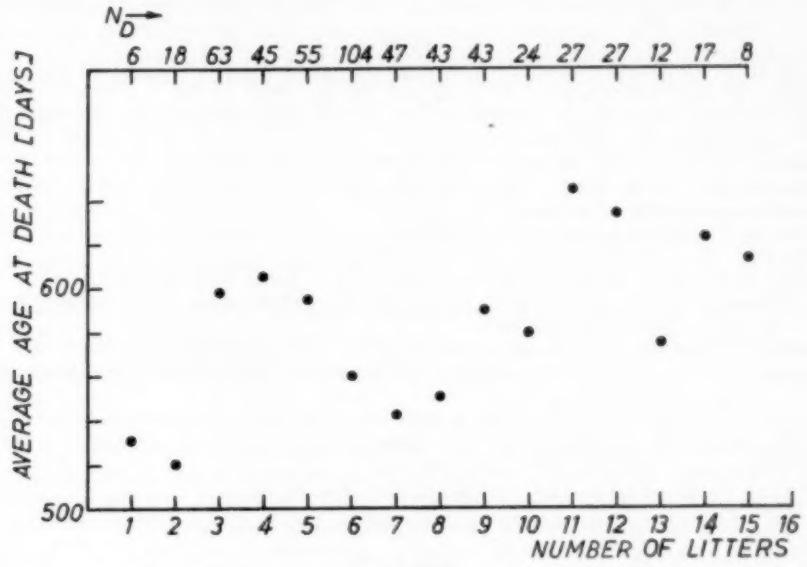


FIG. 5.—Ordinate : Average age at death in days of RIIIf breeders.
Abscissa : Number of litters.

of litter number and/or to the effect of age upon the incidence of mammary tumours.

SUMMARY

1. 108 C_3H_f and 544 $RIII_f$ breeding females have been observed. The number of litters each female had and her survival age or the date of appearance of a mammary carcinoma have been recorded. There were 28 C_3H_f and 14 $RIII_f$ mammary carcinomas. The incidences of tumours of other sites have been included.

2. It was not found possible to draw any conclusions as to the effect of litter number and/or the effect of age upon the incidence of mammary tumours.

Examinations for cytoplasmic hepatoma inclusions were made by H. M. Laird.

REFERENCES

AGNEW, L. R. C. AND GARDNER, W. U.—(1952) *Cancer Res.*, **12**, 757.
ANDERVONT, H. B.—(1950) *J. nat. Cancer Inst.*, **11**, 581.
Idem AND DUNN, T. B.—(1952) *Ibid.*, **13**, 455.
Idem AND MCELENEY, W. J.—(1941) *Ibid.*, **1**, 737.
BURNS, E. L. AND SCHENKEN, J. R. (1943) *Cancer Res.*, **3**, 691.
CLYDE, J. D., LAW, L. W. AND DUNN, T. B.—(1959) *J. nat. Cancer Inst.*, **23**, 717.
DUNN, T. B.—(1953) *Ibid.*, **14**, 1281.—(1959) in ' Physiopathology of Cancer ', edited by Homburger, F. and Fishman, W. H. London (Cassel and Co. Ltd.), 2nd edition, p. 38.
FOULDS, L.—(1956) *J. nat. Cancer Inst.*, **17**, 701.
HEAD, M. A. AND LAIRD, H. M.—(1956) *Rep. Brit. Emp. Cancer Campgn*, **34**, 282.
HESTON, W. E.—(1958) *Ann. N.Y. Acad. Sci.*, **71**, 931.
Idem AND DERINGER, M. K.—(1952) *J. nat. Cancer Inst.*, **13**, 167.—(1953) *Proc. Soc. exp. Biol., N.Y.*, **82**, 731.
Iidem AND DUNN, T. B.—(1956) *Ibid.*, **16**, 1309.
Iidem AND LEVILLIAN, W. D. (1950) *Ibid.*, **10**, 1139.
HUMMEL, K.—(1958) *Anat. Rec.*, **132**, 281.
JONES, E. E.—(1940) *Amer. J. Cancer*, **39**, 94.
MÜHLBOCK, O.—(1950) *J. nat. Cancer Inst.*, **10**, 1259.
PREHN, R. T., MAIN, J. M. AND SCHNEIDERMAN, M.—(1954) *Ibid.*, **14**, 895.
PULLINGER, B. D.—(1952*a*) *Brit. J. Cancer*, **6**, 69.—(1952*b*) *Ibid.*, **6**, 78.—(1955) *Ibid.*, **9**, 613.—(1959) *Ibid.*, **13**, 99.
PYBUS, F. C. AND MILLER, E. M.—(1940) *Amer. J. Cancer*, **40**, 47.
SHIMKIN, M. B.—(1945) in a Symposium on Mammary Tumors in Mice. *Amer. Ass. Advanc. Sci., Wash.*, p. 85.
SIMONDS, J. P. (1925) *J. Cancer Res.*, **9**, 329.
VAN RŸSSEL, T. G. AND MÜHLBOCK, O.—(1955) *J. nat. Cancer Inst.*, **16**, 659.
WHITEHEAD, R.—(1932) *J. Path. Bact.*, **35**, 415.
WOOLLEY, G. AND LITTLE, C. C.—(1945) *Cancer Res.*, **5**, 193

ADDENDUM.

Thanks to the kind collaboration of Professor M. G. P. Stoker and Dr. M. Sussman of the M.R.C. Virology Unit, Glasgow, sample sera from $RIII_f$ and C_3H_f mice have been tested for polyoma antibody. Hæmagglutination inhibition titres over 1/320 were found in both strains.

TESTS FOR MAMMARY TUMOUR AGENT IN C_3H_f AND $RIII_f$ MOUSE STRAINS

B. D. PULLINGER

From the Cancer Research Department, Royal Beatson Memorial Hospital, Glasgow

Received for publication February 29, 1960

REFERENCE data for eventual comparison of the spontaneous incidence of mammary carcinoma in C_3H_f/Pu female mice (Pullinger and Iversen, 1960) with that to be seen after substitution of graded amounts of ovarian oestrogenic hormones have been recorded. Before these substitutions could be undertaken it was also necessary to examine this strain for presence or absence of the mammary tumour agent discovered by Bittner. No evidence of infection with the agent was found among the forebears by Heston and his colleagues (Heston, 1958), nor has any been detected in this laboratory. Evidence has been sought for in-breeding records, from injections of spleen and tumour extracts, by attempts at forced activation of the pathogenic action of the agent and by cross-suckling new-born $RIII_f$ mice on C_3H_f nursing mothers.

MATERIALS AND METHODS

The source, management and methods of examination of the the two strains have been described (Pullinger and Iversen, 1960).

Detection of the mammary tumour agent

If the agent is present in small amount the infection may not be revealed by mammary carcinoma in one generation of mothers but only in breeders of succeeding generations (Andervont, 1950). Thus the most reliable biological criterion for judging absence of agent is considered to be failure to find an accumulation of mammary carcinomas in sublines in breeders observed through several generations (Heston, 1958). When the agent is present in greater amounts, whether by natural infection or by injection of extracts of tissues or tumours, its pathological action is readily revealed in susceptible agent-free females after breeding. No objective measure of the amount of agent is available. The origin and pedigree records of the mice used for testing must be free of suspicion that they themselves are carrying this agent. The $RIII_f$ strain has been used for this purpose, hence the need to examine recent breeding records.

Since Bagg's functional or force-breeding test was introduced (Bagg and Jacksen, 1937) it has been considered necessary by some workers either to force-breed the mothers in order to cause milk stagnation, or, by others, to allow numerous normal pregnancies (Mühlbock, 1950; Mühlbock, van Ebbenhorst Tengbergen and van Rijssel, 1952) because these conditions are believed to be the most favourable for induction of mammary carcinoma. Neither Little and Pearson (1940) nor Bittner (1948) were able to confirm the favourable influence of force-breeding. A direct relationship between increase in number of pregnancies with normal lactation or survival age and mammary tumour incidence appears to be questionable (Pullinger and Iversen, 1960) but no better standards for measuring incidence are yet available. Therefore the practice of rapid breeding has been

followed in the experiments here recorded and only those females that had 3 or more litters have been included in the results.

No electron microscope studies have been made. As no biological evidence of the agent had been found it was unlikely that virus particles would be plentiful and therefore readily detected or, if seen, that they would necessarily represent Bittner's mammary tumour agent. Nor has any attempt been made to increase the amount of this agent by culture in cells *in vitro* in these laboratories. No account of such studies elsewhere has been found. It is not known whether this branch of the original C_3H strain carries the polyoma agent. No spontaneous mammary tumours similar to those caused by the polyoma agent and described by Clyde, Law and Dunn (1959) or by Stanton *et al.* (1959) have been observed in this colony nor in any of the experimental mice.

The natural occurrence and forced production of adenomas (hyperplastic nodules) to detect the mammary tumour agent in young mice were investigated. To avoid repetition these observations and experiments are recorded with their results.

RESULTS

RIII$_f$ breeding records and mammary carcinoma

An incidence of this tumour of 12 in 482 breeders or 2·5 per cent in the first 34 generations has been recorded (Pullinger, 1955). Among 544 breeders in generations F.35 to F.52 it was 14 or 2·6 per cent (Pullinger and Iversen, 1960). In the F.35 generation 6 mammary adenocarcinomas arose in 6 out of 67 breeders thus giving a higher incidence than had ever been found before. This rise might have been due to increase in a persisting small amount of agent or to its more recent acquisition. The attempt was made to breed from tumour-bearing females in F.35 to look for tumour increase in sublines. As mammary carcinoma arose so late in life at an average age of 19·6 months in these 6 females, it was by chance that 1 subline, derived by direct descent, and 2 sublines, derived from siblings of other tumour bearers, had been bred when the unusual high incidence was noted. All 6 tumour-bearing females belonged to different sublines in F.35, having a common ancestor far back in F.24. Neither this common ancestor nor any intervening progenitors had developed overt mammary carcinoma but the mother in F.34 of one of the 6 tumour bearers in F.35 was found at necropsy and by bulk-staining to have a microscopic adenocarcinoma of Type A (purely acinar) in a first left nipple area. Her daughter developed an anaplastic carcinoma in the second right nipple region at 16 months of age. A biopsy was done and three and a half months later the primary had recurred. At necropsy massive pulmonary metastases were found. Subline I, directly descended from this tumour-bearing female, was bred through 5 generations which included 39 effective breeders that lived to the ages given in Table I. None developed mammary carcinoma. Of the two sublines derived from siblings of 2 of the other 5 tumour-bearing females, one, subline VI, was bred for 9 generations and included 36 effective breeders that lived to the ages given in Table I. No mammary tumour arose in these descendants. The third subline was bred for 2 generations with 7 effective breeders. None developed this tumour. In assessing incidence, although records were kept of all mated females, only those that had more than 2 litters and lived to 8 months or over have been considered effective. None that failed to reach these standards developed mammary carcinoma. From the Table I of Pullinger and Iversen

TABLE I.—*Numbers, Survival Ages and Mammary Carcinoma Incidence in Sublines I and VI and in Experimental RIIIf Breeders of 3 or More Litters*

	Months of age																					Number of breeders	Number with mammary carcinoma
	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	
Subline I	—	—	—	1	5	2	1	2	—	1	1	2	3	1	7	4	—	2	4	—	2	1	39	0
Subline VI	2	2	1	—	1	1	2	—	—	2	1	3	1	3	5	2	7	2	1	—	—	—	36	0
Cross-suckled C_3H_f—																								
Without tumours F.1	—	—	—	—	1	1	—	2	—	—	—	—	2	1	2	2	2	1	4	—	—	—	18	0
With tumour F.1	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	1
Without tumours F.2	—	—	—	—	—	—	—	—	—	1	—	1	—	—	—	2	2	—	—	—	—	—	6	0
Test of C_3H_f spleen extract, without tumours, F.1 and F.2	—	1	—	1	1	—	—	1	6	1	—	—	—	1	—	—	—	—	—	—	—	—	12	0
Test of C_3H_f tumour extract without tumours, F.1	1	—	—	—	—	—	—	—	—	—	1	—	1	—	—	2	1	—	—	—	—	—	6	0
Test of CBA tumour 3—																								
Without tumours	—	—	—	1	1	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	3	0
With tumours	—	1	—	1	3	—	1	—	1	1	—	2	—	—	—	—	—	—	—	—	—	—	10	10

Where no figures are entered there were no deaths.

(1960) it can be seen that the unusually high incidence found in F.35 was not maintained in succeeding generations whether breeding was restricted or not. The survival ages in most later generations was not less than in F.35. Since the reference data and Table I (Pullinger and Iversen, 1960) were compiled, 20 out of 23 and 23 out of 25 breeders in F.53 and F.54 have died without tumours. The remaining 5 are 23 months of age or over and are free of tumours. The instance just recorded is the only one among a total of 26 mammary tumour bearers in 52 generations in which a mother and daughter were found with these tumours, one microscopic and one overt. The great-grandmother of one female in F.37 with a mammary carcinoma also bore this type of tumour. The only siblings found with overt mammary carcinoma were 2 out of 4 in F.49. Once previously in F.13, one sister developed a macroscopic and one a microscopic carcinoma. No other close relationships of tumour-bearing females were found.

C_3H_f Strain

Presumptive tests for mammary tumour agent

While pedigree records were accumulating, some presumptive tests for agent were done.

1. Extracts of spleen derived from surplus C_3H_f males in early litters were made by spinning a 10 per cent suspension of homogenised tissues for 15 minutes at 14,000 g. Intraperitoneal injections of 0·05 ml. of supernatant fluid were made into each of 13 $RIII_f$ females aged 3 to 4 weeks. All were subsequently force-bred. Nine in the F.1 generation and 3 in F.2 survived from 8 to 21 months after bearing 3 or more litters. No mammary carcinoma arose (Table I).

2. An extract was later prepared from a mammary carcinoma in a C_3H_f breeder. Intraperitoneal injection of 0·5 ml. of supernatant fluid from a 10 per cent suspension spun at 14,000 g. were made into 9 $RIII_f$ females aged 3 to 4 weeks. All were subsequently force-bred. Six survived for 8 to 24 months after bearing 3 or more litters. No mammary carcinoma arose (Table I).

3. Twenty-nine $RIII_f$ females were born in the presence of, and were left with 15 C_3H_f nursing mothers. Their own mothers were removed within, at most, 16 hours of birth. These F.1 cross-suckled $RIII_f$ females and 6 of their F.2 progeny were subsequently force-bred. Nineteen of the original 29 bore 3 or more litters and lived for 8 to 26 months. One breeder only of the 19 in F.1 developed a perianal mammary carcinoma of compound organoid type at 9 months of age after bearing 6 litters. Neither of her two C_3H_f foster mothers developed mammary carcinoma. Six only of the pooled F.2 generation were reared and mated. They lived to the ages given in Table I, bore multiple litters, and none developed mammary carcinoma.

4. It was previously reported that adenomatous mammary nodules were found regularly in the mammae of young females under a year old in several strains infected with milk-factor, with the exception of Strain A, but that none was seen in young virgins or breeders under this age after the presumed exclusion of agent (Pullinger, 1952, 1955). Precise figures for these nodules in the original C_3H (Andervont) strain infected with agent are not known. Nodules were observed in two C_3H_b substrains freed from agent in females of 15 months or older but data for younger mice were not given (Jones, 1951). Thus no comparison can be made. Nevertheless a systematic examination of bulk-stained preparations of all

570 nipple areas of 57 C_3H_f virgin females aged 11 months was undertaken. Two hyperplastic nodules were found in 570 nipple areas.

5. Use was made of a previously recorded observation that the pathogenic action of the mammary tumour agent in respect of adenoma induction and sometimes of carcinoma could be stimulated to develop earlier in susceptible, infected, castrate mice of either sex by means of a few large, repeated doses of oestrone or oestradiol but that, using the same technique, none was found in mice of the same strain that had been freed of agent. Twelve C_3H_f females ovariectomised at 2 months of age were given 2 doses of 400 μg. oestradiol in acetone *per cutem* at an interval of 20 days. At 8 months of age the 11 survivors were killed and all nipple areas bulk-stained and examined. Regression of physiological response had occurred in all and no carcinoma or adenoma was found. When a similar test was done on 10 C_3H females (derived from another subline) infected with agent, adenomas were found in all 10 (Pullinger, 1947). Similarly 4 $RIII_f$ males injected with spleen extracts and 13 males nursed with their sisters by C_3H_f mothers were castrated, treated with 3 doses of 400 μg. oestrone and 1 dose of 400 μg. progesterone, and were killed at 8 months of age and all fat pads examined by bulk-staining. No adenoma or carcinoma was found. In previous similar tests of RIII males carrying mammary tumour agent, adenomas were found in 28 and carcinoma in 9 out of 30 mice but in none out of 18 $RIII_f$ males (without agent) similarly treated (Pullinger, 1947). The susceptibility of the $RIII_f$ mice to agent was again tested intraperitoneally with CBA tumour extract. Ten out of 13 $RIII_f$ sucklings developed mammary carcinoma after they had been force-bred (Table I). This CBA subline had previously been found to carry the agent (Pullinger, 1953).

C_3H_f breeding records and mammary carcinoma

The overall incidence was 25·9 per cent (Pullinger and Iversen, 1960). These tumours were distributed haphazard in sublines. Records including 3 generations of ancestors of 26 out of 28 of the tumour-bearing females were available for pedigree analysis. The records of the great-grandmothers of 2 were unknown owing to transfer of the original breeding litter to this laboratory, but neither their mothers nor grandmothers had mammary carcinoma. None of the 26 remaining tumour bearers had 3 ancestors with these tumours in the direct line of descent. Five pairs of mothers and daughters had tumours of this kind and one of the latter had also a grandmother with mammary carcinoma. The only other tumour-bearing relations were 8 females with great-grandmothers and 4 with grandmothers which had borne these tumours. The remaining 9 (about a third) had no known progenitor with this carcinoma. These data reveal no evidence of accumulation of mammary carcinoma in sublines.

COMMENT

Breeding records through 11 generations of C_3H_f and 52 generations of $RIII_f$ female mice have failed to provide evidence of an overall increase or an accumulation of mammary carcinomas in any sublines such as is known to occur when the mammary tumour agent is present initially even in small amount. In the combined tests for presumptive evidence of the agent in C_3H_f mice 1 mammary carcinoma arose in 43 $RIII_f$ test females that had been force-bred. Two adenomatous (hyperplastic) nodules were found in 570 nipple regions of 57 C_3H_f virgin females aged

11 months. These two observations, namely 1 mammary carcinoma in a young cross-suckled breeder and 2 nodules in young virgin females, provide the only evidence that could be construed as indicating that the mammary tumour agent was responsible for all or part of the mammary carcinoma incidence in the C_3H_f strain. The single adenocarcinoma in 43 $RIII_f$ test mice could have arisen " spontaneously " independently of the experimental procedures. However, it arose earlier, at 281 days, than any previously found in $RIII_f$ breeders. The range for the first 12 carcinomas in F.1 to F.34 was 389 to 753 days. In the present generations, F.35 to F.52, two of 14 adenocarcinomas were found at 327 and 330 days of age. Thus with larger numbers of tumour-bearing mice a wider spread in age incidence became evident. This seems a more likely explanation of the earlier occurrence of this single carcinoma than that one only out of 43 $RIII_f$ test mice was infected by an agent derived from the C_3H_f strain.

The value of the observation of 2 adenomatous nodules at 11 months of age in 570 nipple regions cannot be assessed without further data about the earliest age when they appear in this strain. In the $RIII_f$ mice none has been seen before one year of age (Pullinger, 1952, 1955).

SUMMARY

1. Pedigree records of 11 generations of C_3H_f and of the last 18 generations of $RIII_f$ breeding females have failed to provide evidence of an accumulation of mammary carcinoma on sublines.

2. No evidence of the mammary tumour agent of Bittner was found in tests with injected spleen or tumour extracts nor any indisputable evidence among susceptible agent-free cross-suckled young mice.

3. Experiments designed to stimulate the pathogenic activity of the mammary tumour agent failed to reveal evidence of its presence.

REFERENCES

Andervont, H. B.—(1950) *J. nat. Cancer Inst.*, **11**, 545.
Bagg, H. J. and Jacksen, J.—(1937) *Amer. J. Cancer*, **30**, 539.
Bittner, J. J.—(1948) *Cancer Res.*, **8**, 625.
Clyde, J. D., Law, L. W. and Dunn, T. B.—(1959) *J. nat. Cancer Inst.*, **23**, 717.
Heston, W. E.—(1958) *Ann. N.Y. Acad. Sci.*, **71**, 931.
Jones, E. E.—(1951) *Cancer Res.*, **11**, 260.
Little, C. C. and Pearson, J.—(1940) *Amer. J. Cancer*, **38**, 224.
Mühlbock, O.—(1950) *J. nat. Cancer Inst.*, **10**, 1259.
Idem, van Ebbenhorst Tengbergen, W. and van Rijssel, Th. G.—(1952) *Ibid.*, **13**, 505.
Pullinger, B. D.—(1947) *Brit. J. Cancer*, **1**, 177.—(1952) *Ibid.*, **6**, 69.—(1953) *Ibid.*, **7**, 490.—(1955) *Ibid.*, **9**, 613.
Idem and Iversen, S.—(1960) *Ibid.*, **14**, 267.
Stanton, M. F., Stewart, S. E., Eddy, B. E. and Blackwell, R. H.—(1959) *J. nat. Cancer Inst.*, **23**, 1441.

Addendum (24.5.60).

Thanks to the kind collaboration of Professor M. G. P. Stoker and Dr. M. Sussman of the M.R.C. Virology Unit, Glasgow, sample sera from $RIII_f$ and C_3H_f mice have been tested for polyoma antibody. Hæmagglutination inhibition titres over 1/320 were found in both strains.

EXPERIENCES WITH HYPOPHYSECTOMY IN MICE.

Criteria of Complete Removal

STRETTON YOUNG and LUCY E. FRASER

From the Clinico-pathological Laboratories of the Imperial Cancer Research Fund, London, W.C.2

Received for publication February 3, 1960

It is important to users of hypophysectomised mice to know if removal of the pituitary gland has been complete. Information on this point may be obtained by *post-mortem* examination of each pituitary fossa, carried out either by direct inspection using a relatively low power microscope with reflected light, or by cutting serial histological sections of the entire area after decalcification and paraffin embedding. The results of examining 432 mice by the latter method have already been reported (Young, 1959).

Although the histological methods described permit the recognition of very small fragments, they are both laborious and time-consuming and it would be of great benefit if other criteria, more readily available, could be shown to give equally useful results. Indirect evidence may be obtained by observing and measuring the effects of hormone withdrawal on the appropriate target organs. Thus, somatic growth, if still occurring at the time of complete removal, will cease thereafter, and the mammary glands, gonads, adrenals and other target organs will undergo progressive atrophy. The response of the mammary glands is of particular interest in this laboratory, for the Mammogenic Growth Response in hypophysectomised mice has been used in investigations into the occurrence of growth promoting substances in normal female urine.

Griffiths (1941) has used the criteria of " cessation of growth, extreme atrophy of adrenals and testes and absence of macroscopic pituitary fragments ". Lostroh and Jordan (1955) examined the adrenals, ovaries, and uteri both macroscopically and by weighing. The pituitary fossa was examined with a " binocular microscope " but sections were not cut. Bahner and von Graff (1957), after cutting serial sections of 25 mouse heads, considered that " remnants of the hypophysis are rather reliably indicated by gonadal weight " ; they particularly favoured seminal vesicle weight.

It is the purpose of this paper to compare several of these criteria with the histological findings after cutting serial sections of over 200 mouse heads, and to consider whether any of them offer a satisfactory alternative to the latter. We believe that a detailed comparison of this nature has not previously been reported.

MATERIAL AND METHODS

Hypophysectomy was carried out on 207 weanling male A_2G mice using the technique described by Thomas (1938). The animals were aged between 22 and 27 days and most of them weighed between 10 and 14 g. ; a few were as light as 9 g. or as heavy as 15 g. After operation they were fed on Diet 41B with rolled

oats and water *ad libitum*. No glucose or antibiotic was given. The mice were weighed daily for 14 days and at progressively longer intervals thereafter, if allowed to survive.

The technique used in killing, fixing and staining pelts has been described (Hadfield and Young, 1956). The stained and mounted glands were examined for the presence of " clubs ", rounded, densely staining structures found at the ends of growing ducts and having a diameter more than twice that of the duct from which they arise. They consisted of closely-packed epithelial cells, many of which were in mitosis. Enumeration of clubs therefore gave an estimate of the degree of mitotic activity and hence a measure of the rate at which new duct formation was taking place. The mean number of " clubs " per mouse was regarded as the Mammogenic Growth Response (M.G.R.).

The heads of the mice were fixed, decalcified, trimmed, embedded and cut serially. Residual fragments of pars anterior were recorded as size + if only a few cells were found ; size ++ if large enough for typical histological structure to be recognised, or size +++ if they amounted to a substantial fraction of the whole gland (Young, 1955). In view of the possibility that pituitary fragments might be transplanted into the brain at operation, the pituitary fossae of 63 of the mice were examined serially without removing the brain.

TABLE I.—*Details of Treatment Given to* 207 A_2G *Mice, Hypophysectomised at ages between* 22 *and* 27 *Days whose Heads have been Examined Histologically for Residual Pituitary Fragments*

Number of mice	Nature of injection	Dose : per mouse per 5 days	Days : Operation	Days : Death
86	None	Nil	7	142
21*	,,	,,	8	15
43	Oestrone + progesterone	1·25 μg. + 7·5 mg.	8	19
23	Prolactin	50 i.u.	7	16
34	Growth hormone	0·2 mg.–1·0 mg.	7	14

* In these 21 mice the operation was intentionally incomplete.

Most of the mice had been used as controls in experiments on the mammotrophic activity of urines and hormones. The details of injections of all mice whose heads were cut histologically are given in Table I. After fixation of the carcasses of the 107 uninjected mice, the organs under investigation were dissected out as follows :

Testes	in 69 animals
Seminal vesicles	,, 68 ,,
Adrenals	,, 69 ,,

The tissues were lightly blotted on filter paper and weighed on 0–10 mg. or 0–50 mg. torsion balances. A further 169 hypophysectomised mice were used in preparing Tables IV and V. The heads of these were not cut histologically.

RESULTS

Somatic weight

Since our mice were hypophysectomised while they were growing rapidly, one of the most noticeable effects of operation was the cessation of growth. This is

shown in Table II and Fig. 1 in which the mean weights of completely hypophysectomised and intact animals have been compared for a period of 7 days. In Fig. 2 the mean weights of a smaller number of mice have been followed for 21

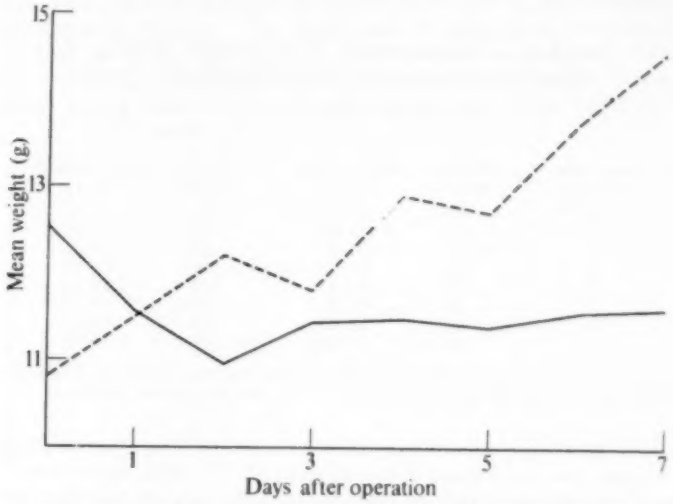


FIG. 1.—Comparison of mean somatic weights of hypophysectomised and intact mice over 7 days.
— — — — Intact mice. ———— Hypophysectomised mice.

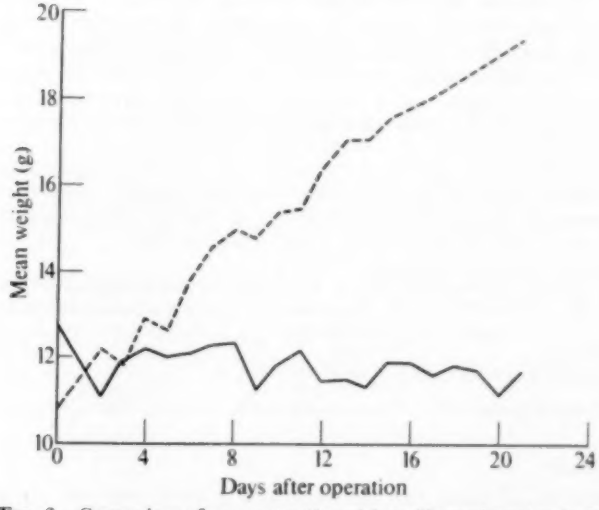


FIG. 2.—Comparison of mean somatic weights of hypophysectomised and intact mice over 21 days.
— — — — Intact mice. ———— Hypophysectomised mice.

days. It is to be noted that the mean weight of completely hypophysectomised mice did *not* return to its pre-operational level although the weights of individual mice have sometimes done so. This was equally true over both short and long periods of time.

TABLE II.—*Comparison of Mean Somatic Weights of Hypophysectomised and Intact Mice Over 7 Days*

	Hypophysectomised mice		Intact mice	
Days after operation	Number of mice	Mean weight (g.)	Number of mice	Mean weight (g.)
0	69	12·53	20	10·8
1	67	11·56	20	11·5
2	43	10·95	20	12·2
3	36	11·42	20	11·8
4	60	11·47	20	12·9
5	64	11·38	20	12·7
6	60	11·53	20	13·7
7	68	11·58	20	14·5

Varying numbers in the second column are due to the fact that these mice belonged to several different groups and daily weighings were omitted on Sundays and public holidays.

To test whether fragments of pars anterior secreted enough growth hormone to influence weight, the individual body weights of 177 mice were examined. The mice were classified according to the histological size of the fragment found. Thus, 124 were completely hypophysectomised; 32 had fragments of size +; while a further 21, intentionally incomplete removals, had fragments of size +++.

The percentage gain or loss of weight 7 days after operation was compared in these three groups and the results are given in Table III. From this it can be seen that the mean loss of weight in completely hypophysectomised mice was greater than in those shown to possess size + fragments and this difference ± standard error was statistically significant ($P < 0.05$). In the case of mice with size +++ fragments there was a mean *gain* in weight instead of a loss and this difference ± standard error was very highly significant ($P < 0.001$).

TABLE III.—*Data Relating to Percentage Somatic Weight Gain or Loss 7 Days After Operation between Groups of Mice*: (a) *Completely Hypophysectomised*; (b) *with Residual Fragments of Pars Anterior Size* +; (c) *with Residual Fragments of Pars Anterior Size* +++

	Without remnants	With histological pituitary remnants (Size +)	With histological pituitary remnants (Size +++)
Number of mice	124	32	21
Mean % weight gain or loss in 7 days	−6·19	−1·95	+13·555
Standard error of mean	0·432	1·732	2·958

Mammary glands

The possibility of using the mammogenic growth response of hypophysectomised mice, treated with oestrone and progesterone, as a measure of the secretion of " mammotrophin " by fragments of pars anterior was considered. Seventy-

eight hypophysectomised mice were divided at random into six groups of 13 each (3 died during the experiment). All the mice were given oestrone + progesterone in oil twice daily for 5 days, to a total dose of 1·25 μg. and 7·5 mg. respectively. In addition, each group was injected twice daily with a solution of prolactin in saline over the same period. The total doses for each group ranged from 0·0008 mg. to 2·5 mg. per mouse per 5 days. The animals were killed and skinned on the sixth day and the mammogenic growth response estimated. The experiment was repeated giving doses of growth hormone ranging from 0·000032 mg. to 0·5

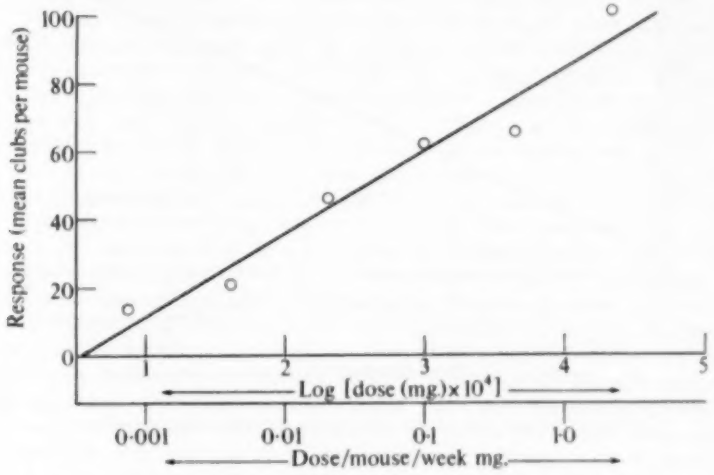


FIG. 3.—Regression of mammary growth response on dose of prolactin in hypophysectomised mice treated with oestrone and progesterone.

mg., instead of prolactin. The results are given in Tables IV and V and in Fig. 3 and 4. It appears that the mammogenic growth response in hypophysectomised mice treated with steady doses of oestrone and progesterone together with variable doses of prolactin or growth hormone was proportional to the logarithm of the doses of the latter, for in both cases linear regression was

TABLE IV.—*Data Relating to Regression of Mammogenic Growth Responses on Dose of Prolactin in Hypophysectomised Mice Treated with Oestrone and Progesterone* 1·25 *μg. and* 7·5 *mg. per Mouse per* 5 *Days*

Prolactin (mg./mouse/5 days)	Mammary growth response (Mean clubs/mouse)	Number of mice
0·0008	13·76	13
0·004	20·53	13
0·02	46·77	13
0·1	61·92	13
0·5	65·75	12
2·5	101·45	11

Linear regression is highly significant $P < 0·001$.

significant ($P < 0{\cdot}001$ and $P < 0{\cdot}01$ respectively). In incompletely hypophysectomised mice treated with oestrone and progesterone alone, it might therefore be possible to use the mammogenic growth response as a guide to the presence and amount of the circulating mammotrophic hormones which have been excreted by the residual pituitary fragments.

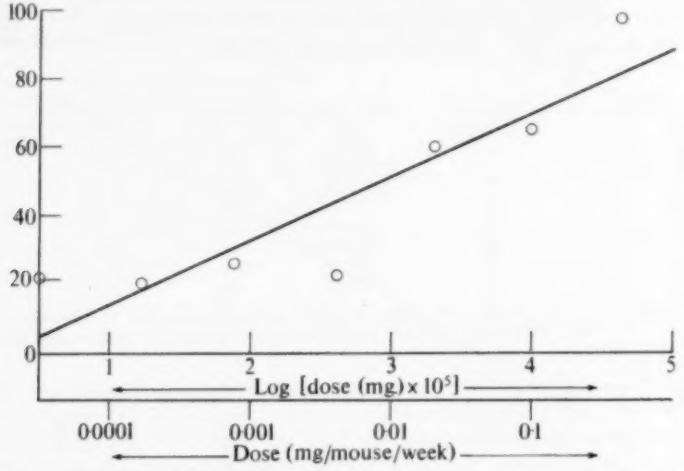


FIG. 4.—Regression of mammary growth response on dose of growth hormone in hypophysectomised mice treated with oestrone and progesterone.

TABLE V.—*Data Relating to Regression of Mammogenic Growth Responses on Dose of Growth Hormone in Hypophysectomised Mice Treated with Oestrone and Progesterone, 1·25 μg. and 7·5 mg. per Mouse per 5 Days*

Growth hormone dose (mg./mouse/5 days)	Mammary growth response (Mean clubs/mouse)	Number of mice
0·000032	21·00	13
0·00016	20·61	13
0·0008	25·53	13
0·004	22·84	13
0·02	59·76	13
0·1	65·07	13
0·5	97·46	13

Linear regression is statistically significant $P < 0{\cdot}01$.

Forty-three hypophysectomised mice injected with oestrone and progesterone in arachis oil were examined histologically for residual fragments of pituitary. The total dose of oestrone was 1·25 μg. and of progesterone 7·5 mg., one-tenth of this being given at each injection, night and morning, for 5 days. Nine mice were shown to have residual fragments of pars anterior, all of them size +.

The mean mammogenic growth response ± standard error in these mice was 6·78 ± 4·3 compared with 4·74 ± 1·401 in the remaining 34 which had no demonstrable pituitary fragments. The difference was not statistically significant

($P = 0{\cdot}1$). Each of these groups could be divided up, however, depending on whether the first injection was given 6 or less, or 7 or more, days after operation. In the latter case, the 6 mice having fragments of size + had a mean mammogenic growth response of $2{\cdot}5 \pm 1{\cdot}453$ while the remaining 22 with no demonstrable fragments had a mean mammogenic growth response of $1{\cdot}36 \pm 0{\cdot}520$. Once again the difference was not statistically significant ($P > 0{\cdot}2$).

A comparison was also made between mammogenic growth responses of *completely* hypophysectomised mice (*a*) having their first injection 6 days or less after operation (12 mice, mean M.G.R. $= 10{\cdot}92 \pm 3{\cdot}219$) and (*b*) having their first injection 7 days or more after operation (22 mice, mean M.G.R. $= 1{\cdot}36 \pm 0{\cdot}520$). The mice having a first injection a week or more after operation gave consistently low mammogenic growth responses, while those injected within a few days of operation not only gave high mammogenic growth responses but showed greater variation from one mouse to another. The difference between the two groups was very highly significant ($P < 0{\cdot}001$). It is possible that this difference might be due to the incomplete excretion or destruction of the mamotrophic hormone(s) during the first few post-operative days. If this is so, it means that in determining mammotrophic activity, false positives are liable to occur if hypophysectomised mice are used too soon after operation.

There is yet another way in which the mammary gland might be used in trying to assess the functional activity of fragments of anterior pituitary. Since the secretion of oestrogenic and progestational steroids is under the control of the pars anterior, and since oestrone + progesterone + impure prolactin has been shown to be actively mammogenic, it may be possible to estimate gonadotrophin activity indirectly by means of the mammogenic growth response in hypophysectomised mice treated with prolactin alone. Twenty-three hypophysectomised mice treated with 50 i.u. prolactin per mouse per 5 days were examined histologically for evidence of pituitary fragments. Fragments of size + were found in 6 mice. These had a mean mammogenic growth response of 1·16 compared to 1·35 for the 17 mice in which no fragments were found. It is clear that no measurable gonadotrophin activity could be detected by this method in mice with fragments of size +.

Testis and seminal vesicle weights

The direct measurements of gonadotrophin excretion in mice is not practicable but activity may be assessed indirectly by examination of the appropriate target organs. Sixty-nine mice were available for study. At death 7–16 days after operation, the testes and seminal vesicles were taken for weighing. In this group the 42 animals completely hypophysectomised had a mean testis weight ± standard error of 29·54 mg. ± 2·55 mg. with a mean seminal vesicle weight (41 animals) of 1·84 mg. ± 0·083 mg.; the 6 mice with size + fragments had a mean testis weight of 39·32 mg. ± 12·424 mg. with a mean seminal vesicle weight of 2·27 mg. ± 0·249 mg.; while the 21 mice with size +++ fragments (intentionally incomplete operations) had a mean testis weight of 74·92 mg. ± 7·83 mg., with a mean seminal vesicle weight of 19·11 mg. ± 3·166 mg.

In animals with size + fragments, testis weights did not differ significantly from those in completely hypophysectomised mice. Seminal vesicle weights, however, showed a greater difference. If it is admitted that complete hypophysectomy could only lead to a reduction in target organ weight, then the value of

$P < 0{\cdot}1$ could be halved to $P < 0{\cdot}05$, which is statistically significant. In mice with size +++ fragments both testis and seminal vesicle weights showed differences which were highly significant ($P < 0{\cdot}001$ in both cases).

Adrenal weights

Just as deprivation of gonadotrophin gives rise to atrophy of testes and seminal vesicles, so deprivation of adrenocorticotrophic hormone leads to atrophy of the adrenals. As in the examination of testis weights, 69 mice were available for the adrenal study. Forty-two completely hypophysectomised animals had a mean weight ± standard error of 1·17 mg. ± 0·022 mg.; 6 mice with size + fragments had a mean adrenal weight of 1·22 mg. ± 0·054 mg.; while the 21 mice with +++ fragments had a mean adrenal weight of 2·30 mg. ± 0·096 mg. No significant difference in mean adrenal weights was found between mice possessing size + fragments and those with none ($P > 0{\cdot}2$); however, there was a highly significant difference in the comparison of mice with size +++ fragments and those containing none ($P < 0{\cdot}001$).

DISCUSSION

From a consideration of these results it is clear that the presence or absence of size +++ fragments was associated with very significant differences in somatic weight gain or loss, and also with significant differences in the weights of testes, seminal vesicles and adrenals. Unless they were left behind deliberately, fragments of size +++ were very uncommon. Their identification was, therefore, relatively unimportant but their presence was detected by daily weighings of the operated mice, since animals gaining weight regularly after the first few post-operative days have been found to possess fragments of this size (Fig. 5).

The discovery of mice with size + fragments was a more important problem, for these occurred more frequently. It appears that significant differences in somatic and seminal vesicle weights were present between mice containing size + fragments and those with none. The seminal vesicle weight difference was found a satisfactory indicator by Bahner and von Graff (1957) but it has the disadvantage that it entails either another operation, or death of the mouse. In the appraisal of mice for experiment the advantages lie clearly with selection by somatic weight. Unfortunately, the 156 mice we have studied in this connection, were too few in number to permit an accurate forecast of the level of weight increase to be confidently associated with the presence of size + fragments. It has become our habit, however, to discard as unsuitable any mouse exceeding its operation weight by 10 per cent on the seventh post-operative day.

Size + fragments appear to have been insufficiently active biologically to give a significantly increased mammogenic growth response with injections of oestrone and progesterone and this has apparently also been true of injections of prolactin given alone. Since fragments of size + are virtually the only size we have found in our mice, this implies that it is no longer necessary for us to cut serial sections of pituitary fossae from hypophysectomised mice treated with oestrone and progesterone, and used in the determination of mammogenic growth responses.

Occasionally no pituitary fragments were found in a mouse which appeared biologically to show evidence of pituitary activity as judged by weight gain following the operation, or a higher mammary growth response than was expected

following oestrone + progesterone administration. There are several possible explanations :

1. The mouse might have been undernourished or partly dehydrated at the time of operation and gained weight subsequently to a normal level.

2. Fragments of pars anterior might have been left behind which continued to secrete hormones for a short time but did not survive permanently. Such fragments have, in fact, been occasionally observed (Young, 1959).

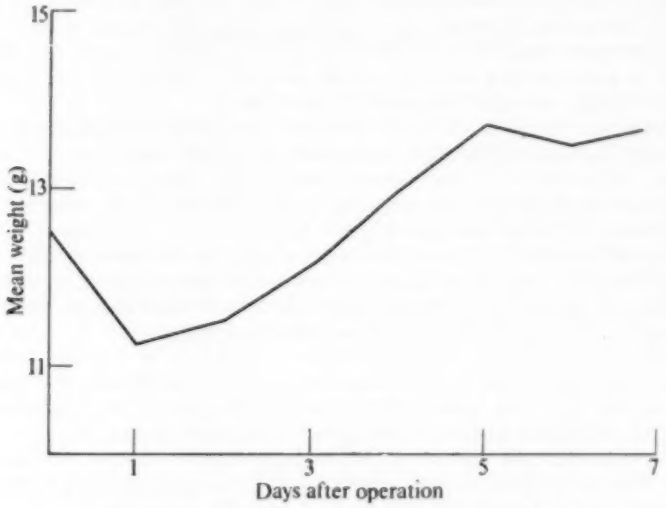


FIG. 5.—Mean somatic weight gain in " hypophysectomised " mice with fragments of pituitary, size + + +.

3. At the time of operation, viable fragments of pars anterior might have been transplanted into the brain, survived there and continued to secrete. Since our usual technique of serially cutting the pituitary fossa involved removal of the brain before embedding, it was unlikely that any such fragments would be found at subsequent examination. To test this possibility, serial sectioning was carried out on 63 mice without prior removal of the brain. Although fragments of pars anterior (+ and + + +), pars intermedia and pars posterior were present in some of these mice, in no single instance was a pituitary fragment found outside the pituitary fossa. Leaving the brain in place made an already tedious procedure more difficult, for the pituitary fossa could no longer be directly observed. The block of tissue had to be trimmed thicker than usual and many more sections were required to cut through it.

4. Finally, there was the possibility of the presence of a biologically active ectopic embryonal remnant. We have no information of its occurrence in mice.

It is interesting to note that there was no evidence of regeneration of size + fragments up to three months after the operation, and bony regeneration of the removed plate of basi-occiput was incomplete.

SUMMARY

1. Over 200 hypophysectomised mice were examined histologically for the presence of residual fragments of pituitary. The presence or absence of pars anterior fragments was compared statistically with differences in somatic weight ; testis weight ; seminal vesicle weight and adrenal weight, and with the mammogenic growth response after treatment with oestrone and progesterone.

2. The presence or absence of size + fragments was associated with significant differences in somatic and seminal vesicle weights. Differences in testis weight and adrenal weight were not statistically significant.

3. It was not found possible to forecast accurately the presence of size + pars anterior fragments from a consideration of weight alone.

4. Mice with size + pars anterior fragments did not show significantly increased mammogenic growth responses when treated with oestrone 1·25 μg. and progesterone 7·5 mg. per mouse per 5 days.

5. In mice completely hypophysectomised and treated with oestrone 1·25 μg. and progesterone 7·5 mg. per mouse per 5 days the mammogenic growth response was significantly greater if injections were started less than 6 days after operation than if they were started 7 days or more after operation.

We are indebted to Dr. C. C. Spicer, Head of the Division of Statistics of the Imperial Cancer Research Fund, for advice in statistical methods, and to Messrs. J. Gilbert, T. O'Connor and P. V. Sharp for technical assistance.

We are grateful also to the Endocrinology Study Section of the National Institutes of Health, Bethesda, Maryland, U.S.A. for the gifts of growth hormone and prolactin, which have made this work possible.

REFERENCES

Bahner, F. and von Graff, H.—(1957) *Acta endocr., Copenhagen*, **24**, 333.
Griffiths, M.—(1941) *J. Physiol.*, **100**, 104.
Hadfield, G. and Young, S.—(1956) *Brit. J. Cancer*, **10**, 324.
Lostroh, A. J. and Jordan, C. W.—(1955) *Proc. Soc. exp. Biol. N.Y.*, **90**, 267.
Thomas, F.—(1938) *Endocrinology*, **23**, 99.
Young, S.—(1959) *Brit. J. Cancer*, **13**, 208.

CARCINOGENS FORMED IN THE HEATING OF FOODSTUFFS. FORMATION OF 3,4-BENZOPYRENE FROM STARCH AT 370–390° C.

W. DAVIES AND J. R. WILMSHURST

From the Department of Chemistry, University of Melbourne, Australia

Received for publication March 17, 1960

THE present investigation is to determine whether well known detectable carcinogens are formed in normal domestic situations, for example by the heating of foods under conditions approximating to those applying in household cookery. Though the 3,4-benzopyrene (BP) found in the soot obtained in the " smoking " of foods and also in the smoked food itself arises from the wood which produces the smoke (Tilgner and Müller, 1957 ; Dobes, Hopp and Sula, 1954 ; Bailey and Dungal, 1958), in some cases the foodstuff itself can, when heated, give rise to carcinogens. Thus nine polycyclic aromatic hydrocarbons including BP have been detected in coffee soots, though they were not present in the coffee beans before heating (Kuratsune and Hueper, 1958). Moreover in the " char " formed in making " cakes " (U.S.A.) BP is detected by a spectrophotometric method which as used by Kuratsune (1956) is considered completely reliable (Fieser, 1957). Another group of polycyclic hydrocarbons containing BP is present in tobacco smoke though not in the original tobacco (Kennaway and Lindsey, 1958). With regard to the temperature attained, it is estimated (Kuratsune and Hueper, 1958, p. 38) that the coffee beans on roasting may be exposed to a temperature of 540° C., and it seems likely that the wood and tobacco are exposed to even higher temperatures. These materials, and most foodstuffs which are heated, contain polysaccharides ; hence it was decided to examine the products obtained by heating starch, which is present in floury mixtures which are sometimes strongly heated.

A temperature of 370–390° C. was chosen as this is likely to be reached in some cooking operations, e.g. we have found that the surface temperature of toasting bread may reach 390–400° C., for this is the temperature rapidly attained when a mica sheathed thermocouple is placed in the same position as bread in an electric toaster. It may be that some temperatures in cooking are even higher, since Ivy (1955) considers that fats in contact with hot metal may reach 400–600° C. This is presumably the temperature range used by Sarasin (1918) who destructively distilled starch (apparently with a flame), and who claimed toluene, phenols, furans, ketones, fatty acids, and a compound $C_{12}H_{14}O$; however, most of his products seem to have been obtained when a mixture of starch with zinc dust was distilled, i.e. in a reducing atmosphere.

In the present work the starch was pyrolysed in an iron pot (equipped with a distillation head) in a metal bath which was electrically heated at 370–390° C. The aqueous distillate formed at ordinary pressure contained a small amount of tar, part of which was alkali soluble ; the neutral portion after some preliminary

separations gave a crystalline complex with benzotrifuroxan (Bailey and Case, 1958) which is a reagent for aromatic types. The aqueous distillate apparently contained no BP.

The carbonaceous residue in the pot contained much oxygen (Found : O ; 10·1 per cent, 11·0 per cent ; on different samples) and the methylene chloride extract after washing with sodium hydroxide was chromatographed on silica gel and then on alumina to give a mixture of compounds from which on further purification a solution of a material with the fluorescence spectrum of BP was obtained. The large amount of interfering material present precluded the use of spectrophotometric methods. The results we have obtained by the fluorescence method are, however, quite unequivocal.

EXPERIMENTAL

3 : 4-Benzopyrene detection (BP).—A fluorescence method was used. The exciting radiation was essentially 365 mμ, a 4 mm. Chance 0X1 filter being used in conjunction with a mercury vapour lamp. A quartz condenser lens was used to illuminate the cell which was placed near the slit of a Hilger medium spectrograph. A glass train was used in the spectrograph so as to make use of the greater dispersion of glass as compared with quartz at 400 mμ, some three times greater with this instrument ; exposure times were however considerably increased. The cells were U-tubes of Pyrex glass, one arm being 7 mm. and the other 0·5 mm. diameter ; B7 sockets allowed each arm to be stoppered.

It was necessary to deoxygenate the solution before investigating the fluorescence spectrum ; this was done by passing, for ten to fifteen minutes, a slow stream of purified hydrogen through the solution. 0·01 μg./ml. of BP could be readily and positively identified (in the absence of interfering materials 0·001 μg./ml.).

Chemicals.—All organic solvents, chromatographic adsorbents and the aqueous sodium hydroxide used were found to be free from BP. The isohexane was a purified petroleum fraction b.p. 58–62° C. The methylene chloride mentioned in the detailed description of the experimental procedure has sometimes been replaced by benzene. The commercial wheat starch used contained small quantities of sodium chloride, lipids, and traces of protein.

The pyrolysis.—The retort was a steel autoclave vessel, volume about 4 litres and internal diameter 14 cm., of which the head had been replaced by an iron head equipped with a Pyrex condenser. To the retort heated by an electrically heated metal bath at 370–390° (bath temperature) the powdered starch (300 g.) was added all at once, the distillation head at once replaced, and the heating continued for 25–30 minutes by which time the distillation had almost ceased. Thermocouples placed in the starch showed an internal temperature not greater than that of the bath. The retort was rapidly cooled to approximately room temperature (15–20 minutes) by circulating water through a stainless steel coil in the metal bath, and the retort then opened. If opened above 70° C. the contents can spontaneously ignite. The distillate (D), largely aqueous, was worked up separately.

Residue, procedure.—The residue (varying from 98 to 108 g.) was removed from the retort, which was then extracted for an hour with methylene chloride under reflux, cleaned, and used for a fresh operation. The residues from four

experiments were coarsely powdered, and each extracted (Soxhlet) with about 500 ml. of methylene chloride until removal of fluorescence ceased. These four extracts together with the extracts from the retort were combined, extracted thrice with 3 per cent aqueous sodium hydroxide, then with water and then dried ($MgSO_4$). The methylene chloride was distilled off through a short column packed with helices, and gave a residue (about 4 g.) of a light brown oil whose solution in cyclohexane, together with cyclohexane washings of the helices, was chromatographed on activated silica gel. The eluate, though very fluorescent, gave no fluorescent bands on further chromatography, the adsorbent becoming uniformly fluorescent, so much so that the band expected by the addition of even milligrams of BP was completely masked. This difficulty was overcome by simultaneously using two columns which were identical except that one (A) contained the unknown fluorescent material from the carbonised starch, and the other (B) only pure BP. The zone in (B) was a guide in collecting the BP fraction from (A) as experiment with several mg. of BP showed that the materials in (A) only slightly affected the rate of elution of the BP present. This procedure of using columns (A) and (B) was followed in all chromatographic separations. It was found convenient to collect the eluate in relatively small equal fractions (50 ml.).

The appropriate fractions from the silica gel column were combined and concentrated and chromatographed on alumina (cyclohexane), but repeated chromatography on either of these adsorbents failed to separate the BP from the interfering fluorescent material. Most of the latter, however, was removed by chromatography on silica gel containing about 15 per cent of dimethylformamide (isohexane saturated with dimethylformamide). This partitioning was repeated with those fractions suspected of containing BP ; after removing the dimethylformamide by washing with water, and then concentrating, the fluorescent spectrum was determined in isohexane.

Distillate, procedure.—For the BP estimation the methylene chloride extract of the distillate (D) formed in the pyrolysis was worked up exactly as with the residue extract.

A preliminary study of the chemical types present in the methylene chloride extract was also made ; it is a complex mixture. The alkali soluble material was found to contain a mixture of fatty acids (m.p. 56·5–57° C. Found : C, 75·3 per cent ; H, 12·7 per cent ; $C_{17}H_{34}O_2$ requires C, 75·5 per cent , H, 12·7 per cent) which thus appears to correspond to a mixture of palmitic and stearic acids, presumably derived from the lipids present in the starch. Phenols were not detected, and the remainder of this fraction has not been characterised but is partly polymeric. After chromatography on silica gel the neutral fraction yielded as their *p*-nitrophenylhydrazones, a complex mixture of ketones. Purification was hindered by polymerisation , analysis did however indicate a high degree of unsaturation. Aldehydes were present in only very small amount (Schiffs test). In addition a crystalline benzo-trifuroxan derivative (m.p. 180–1° C. Found : C, 57·7 per cent , H. 3·3 per cent , N, 17·7 per cent) was obtained. After decomposition of this complex (by solution in dimethyl formamide and extraction of the desired material into isohexane) a yellow oil was obtained (Found : C, 88·9 per cent , H. 6·9 per cent). A formula of the order of $C_{28}H_{26}O$ (required C, 88·9 per cent ; H, 6·9 per cent) would seem correct. Infrared spectroscopy indicates that this is an unsymmetrical aromatic ether. On chromatography it behaves very similarily, as regards degree of adsorption, to BP.

The compounds not extracted from the aqueous distillate by methylene chloride have not yet been examined.

BP estimation.—A series of BP solutions containing the same amount of interfering material as the unknown was used as a standard. An estimate of the concentration of interfering material was obtained by u.v. absorption spectrophotometry, by weight, and by fluorescence spectrophotometry. The unknown and standard solutions were photographed on the same plate; comparison was by means of a microphotometer. Similar results were obtained when pure BP solutions were used as standards, in this case a peak height to baseline ratio method was used. When positive results were obtained, the fluorescence bands at 404, 410, 419, 427, 433 and 455 mμ were quite distinct.

As a result of several runs, the original aqueous distillate (D), was found to be free of BP, whilst the charred residue contained 0·2 μg./100 g. corresponding to 0·07 μg./100 g. of starch.

That the method has validity was also shown by the addition of 5 μg. of BP to the methylene chloride extract of a pyrolysed starch residue (from 300 g. starch), and approximately 3 μg. of BP was recovered. Since the amount of BP originally present is about 0·2 μg. (see above) this represents a 56 per cent recovery.

The amount of BP found (0·2 μg.) is essentially the same when benzene replaces methylene chloride as solvent, and altogether the total experiment of pyrolysing starch and estimating the BP has been done four times with both " char " and distillate (D) with essentially the same result. Though the distillate (D) is extremely fluorescent no BP has been detected in it, though a 60 per cent recovery was made when 5 μg. of BP was added to it. No BP was detected when the whole procedure was carried out starting by extracting unheated starch with methylene chloride.

DISCUSSION

The yield of BP was very minute, being 0·07 μg./100 g. of starch, much lower than that of Gilbert and Lindsey (1957) who obtained 17 μg. of BP per 100 g. of starch which had been heated to 650° C. This yield is probably little higher than that of Kuratsune (1956) who reported 1 to 7 μg./100 g. of " char ". He used a gas heated asbestos surface.

The fluorescence method of detecting BP is so sensitive that there is danger of contamination from outside sources. Hence a blank experiment with the same amount of starch and treatment with the same amount of solvents as when the starch was heated, was carried out. No detectable amount of BP was present, though less than 0·01 μg. of BP per ml. of BP solution can be identified by the process used. Moreover it is significant that the distillate, when exposed to the same extraction treatment as the residue, gave no detectable BP.

It is unlikely that atmospheric contamination is in question since the work was carried out in a laboratory some 26 ft. above the breezy University grounds and some 400 yards from a busy motor road in a relatively non-industrialised area. The minute amount of BP found, corresponding to 1 g. of BP from about 1500 tons of starch, may have no practical significance. Various fractions of the pyrolysis products have so far failed to show carcinogenicity in the animal tests carried out in the Melbourne University Pathology Department, though adequate tests have not yet been made with the relatively large amounts of apparently

aromatic types which are difficult to separate from the BP. Nevertheless the production of traces of BP at the previously unrecorded low temperature of 370–390° C. may foreshadow the production of larger amounts at the same temperature in actual cooking operations where the starch is exposed to oxidation and also the effect of other foodstuffs mixed with it. Such experiments are being made.

SUMMARY

Commercial starch has been " destructively distilled " at atmospheric pressure and at a temperature of 370–390° C. Air was essentially excluded. The charred residue was found to contain 0·2 μg. of benzopyrene per 100 g. " char ". The significance is unknown.

Thanks are due to Professor A. N. Hambly for advice, and to the Anti-Cancer Council of Victoria for a grant. The microanalyses were carried out by Dr. Zimmermann and his staff.

REFERENCES

Bailey, A. S. and Case, J. R.—(1958) *Tetrahedron*, **3**, 113.
Bailey, E. J. and Dungal, N.—(1958) *Brit. J. Cancer*, **12**, 348.
Dobes, M., Hopp, K. and Sula, J.—(1954) *Csl. Onkol.*, **1**, 254.
Fieser, L. F.—(1957) Separatum Festschrift Arthur Stoll.
Gilbert, J. A. S. and Lindsey, A. J.—(1957) *Brit. J. Cancer*, **11**, 398.
Ivy, A. C.—(1955) *Gastroenterology*, **28**, 345.
Kennaway, E. L. and Lindsey, A. J.—(1958) *Brit. med. Bull.*, **14**, 126.
Kuratsune, M.—(1956) *J. nat. Cancer Inst.*, **16**, 1485.
Idem and Hueper, W. C.—(1958) *Ibid.*, **20**, 37.
Sarasin, J.—(1918) *Arch. Sci. phys. nat.*, **46**, 5.
Tilgner, D. J. and Müller, K.—(1957) *Roczn. Technol. Chem. Zywnosci*, **2**, 21.

INDUCTION OF CANCER OF THE CERVIX UTERI IN RELATION TO THE OESTRUS CYCLE

RACHEL STEIN-WERBLOWSKY

From the Marie Curie Hospital, London N.W.3

Received for publication February 13, 1960

THE pathogenesis of cancer of the cervix has been widely studied but no definite conclusions have as yet been reached. Among the causal agents which have been investigated in the human are early gestation (Runge and Zeitz, 1958), chronic inflammation (Hausdorff, 1955; Guimaraes and Braz, 1958) and genetic factors (Casper, 1955, 1958).

Experimentally carcinoma of the cervix has been induced in mice by the subcutaneous administration of hormones (Gardner *et al.*, 1938) or of a combination of hormones and carcinogenic hydrocarbons (Perry, 1936) and by the intravaginal application of these substances (Reagan, Wentz and Machicao 1955; Scarpelli and von Haam, 1957; Koprowska *et al.*, 1958; and Murphy, 1958). Rats appear to be more resistant to the chemical induction of cervical cancer than mice. von Haam and Scarpelli (1955) quote only one instance of cancer following intravaginal tar painting in a series of 50 rats. Vellios and Griffin (1957) induced cervical cancers in rats by inserting a thread impregnated in dimethylbenzanthracene into the uterine cervix. Glucksmann and Cherry (1958) produced 1 vaginal carcinoma and several sarcomata in addition to vulval tumours by the intravaginal application of the same carcinogen. Thus these authors applied carcinogens either intermittently, by painting the cervix at regular intervals or continuously, by the permanent insertion of a carcinogen impregnated thread (Murphy, 1953). In such experiments, however, no attempt was made to evaluate the possibility of cyclical variations in the response of the cervical epithelium to carcinogens in different phases of the oestrus cycle.

The object of the present communication is to describe the results of some experiments designed to analyse the relative responsiveness of the cervical epithelium to benzopyrene in the oestrus and dioestrus phases of the menstrual cycle in the rat.

MATERIALS AND METHODS

Young female adult white Wistar rat, about 3 months old and 140–150 g. in weight, obtained commercially, were used. The phase of the oestrus cycle (Fig. 1 and 2) was determined by making daily vaginal smears. Small amounts of aqueous methylene blue were injected into the vagina, aspirated and examined microscopically. One group of 65 rats was painted per vaginam with 1 per cent benzopyrene in acetone when found to be in the dioestrus phase and 60 animals were similarly treated when in the oestrus phase. It was found that the menstrual cycle of these animals was not regular and that cycles of different duration occurred in the same animal. As paintings were only carried out in the peak oestrus or

dioestrus period respectively, a certain number of cycles were missed out as a peak may have had occurred at a time when smears were not taken. This particular strain of rats was able to breed up to the age of 2 years and it may be assumed that the menopause occurred at that time or somewhat later. There were no obviously post-menopausal smears in the animals under investigation.

Painting was carried out till the animal died. The number of paintings ranged from 14 to 118, the average being about 35 paintings varying according to each individual cycle and to the length of the life span. The duration of the experiment was from 4 to 26 months, the average being about 17 months.

Painting was carried out by covering the stillette of a lumbar puncture needle with cotton wool, dipping into the benzopyrene solution and inserting it into the vagina as far as it would go. The stillette was rotated several times *in situ* before withdrawal. This resulted in a fairly equal distribution of the carcinogen in the cervical and vaginal epithelium. The animals were allowed to die a natural death, they were only killed when found to be ill or suffering. A post-mortem examination was performed on each rat and the body of the uterus, the cervix and the vaginal cuff removed for histology. At least 3 sections, including anterior cervical lip, lateral fornices and posterior lip were made of each specimen. Other organs were taken for histology when their macroscopic appearance suggested an abnormality.

RESULTS

In 12 out of 65 (12 per cent) and 6 out of 60 (10 per cent) rats painted in dioestrus and oestrus respectively, histology was not available due to cannibalism. The most frequent cause of death was pneumonia (55 rats) and gastro-intestinal infection or haemorrhage (12 animals). Other animals suffered from abscesses at various sites or from inanition. They were either allowed to die a natural death or killed when survival seemed unlikely. The significant abnormalities found in the dioestrus group are shown in Table I and those of the oestrus group in Table II (Fig. 3).

TABLE I.—*Significant Abnormalities Detected at Autopsies of 53 Rats Exposed to Vaginal Benzopyrene Painting During the Dioestrus*

Lesion	Number of paintings	Duration of experiment (months)
Cancer of the cervix	14	8
	39	21
Angio-endothelioma (vagina)	15	11
Polypus cervix	20	8
	33	17
Haemorrhagic cyst ovary	42	19
	33	17
	34	16
Mammary adenofibroma	15	11
	69	17
	33	17
	72	24
	33	21
	7	16
Thyoma	65	17
Leukaemia	27	10
Bile duct carcinoma	7	16
All over average	31	16

TABLE II.—*Significant Abnormalities Detected at Autopsies of 54 Rats Exposed to Vaginal Benzopyrene Painting During the Oestrus Phase*

Lesion	Number of paintings	Duration of experiment (months)
Polypus in horn	77	24
Cyst in horn	37	16
Mammary adenofibroma	53	26
	77	24
All over average	40	17

DISCUSSION

As can be seen, cancer of the cervix developed in 2 out of 53 rats painted during the dioestrus (4 per cent). This is of particular interest because, as mentioned previously (Glucksmann and Cherry, 1958) rats appear to be rather resistant to the chemical induction of cervical cancer by painting. Furthermore 1 rat developed an angio-epithelioma of the vagina. Other abnormalities of interest include 1 case of leukaemia, 1 case of bile duct carcinoma and 6 cases of mammary fibro-adenomata. By contrast no malignant neoplasms either of the cervix or vagina or at distant sites were noted in animals painted during the oestrus phase. From the above data it would appear that painting during the dioestrus phase results in a higher incidence of tumours, both malignant and benign than painting during the oestrus phase. These findings may be due to several factors.

(*a*) The basal and regenerating cells may be particularly sensitive to carcinogens as shown by Breedis (1955) who found that the undifferentiated epithelium of regenerating rabbit skin was highly susceptible to chemical carcinogens. Or, the keratinized epithelium may be resistant to such treatment as shown by Twort and Twort (1936) who failed to produce tumours of the soles of feet of mice that were kept on plates smeared with carcinogenic oil.

(*b*) The duration of exposure of the basal cells, rather than their susceptibility might be a factor in carcinogenesis. This aspect has been investigated by Berenblum, Haran-Ghera and Trainin (1958) with reference to the "hair cycle effect." These authors found that the increased incidence of skin tumours in the mouse when paintings were carried out during the resting phase of the hair cycle was not due to a hypersensitiveness during that particular phase but to a difference in retention of a sufficient concentration of carcinogen. Similarly, the carcinogen may persist in the basal cells of the cervix for a much longer period than in the superficial, keratinized-cells receiving identical treatment.

The "hair cycle effect" might still be enhanced in the case of the genital epithelium. According to Glucksmann (1945) mouse epidermal cells differentiate and are cast off in approximately 21 days whereas the cyclical maturation of the vaginal epithelium of the mouse takes about 5 days (Snell, 1941). Benzopyrene

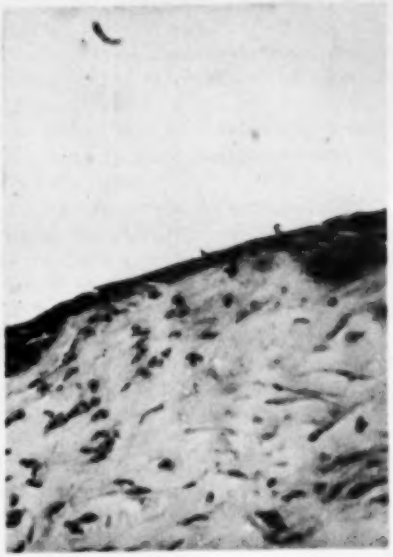
EXPLANATION OF PLATE.

FIG. 1.—Cervical epithelium rat. Oestrus, ×120.

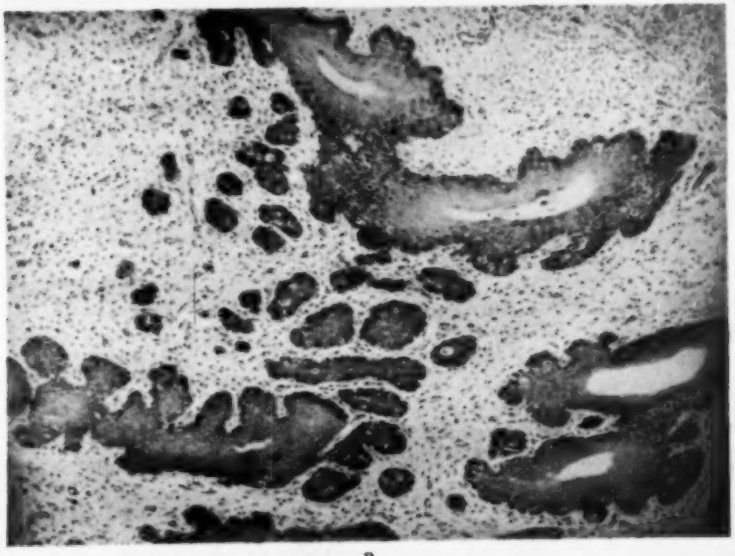
FIG. 2.—Cervical epithelium rat. Dioestrus, ×120.

FIG. 3.—Early cancer of the cervix, ×120 (14 paintings within 8 months).

1



2



3

Stein-Werblowsky.

painting also results in a delay in maturation and an absolute and relative increase in resting cells (Glucksmann, 1945). Thus an ever increasing number of basal cells is exposed to the carcinogen when paintings are given during the dioestrus phase. This, together with the prolonged retention of these substances within these cells, might well result in a greater intensity and duration of exposure (to carcinogens).

By contrast, painting during the oestrus phase affects a relatively smaller number of cornified cells. The period of exposure is also greatly reduced as a number of these cells are cast off within a very short time.

The distant lesions observed in the dioestrus group might be explained by the absorption of the carcinogen by the subjacent blood-vessels of the dermis—a process which is greatly facilitated when the epithelium is of low, dioestrus type. During oestrus absorption may be greatly delayed, as the thick cornified epithelium may form a barrier against the penetration of the carcinogen and as the carcinogen incorporated in the superficial cells is eliminated when these cells are shed.

It may be mentioned in this connection that similar results have been obtained by treating the basal and keratinized cells on other sites. Thus carcinogens applied to the injured gastric mucosa of rats induced 10 cancers among 133 animals whereas application on the intact gastric mucosa did not yield any local gastric tumours among 66 controls, though several distant lesions have been observed (Stein-Werblowsky, unpublished data). This is also in accordance with the findings of Huggins (1958) who induced breast tumours in rats by gastric instillation of methylcholanthrene. No mention is made of a concomitant induction of gastric tumours. Similarly the application of dimethylbenzanthracene on the shaved and injured intrascapular skin in mice yielded 69 warts, 3 carcinomas and 19 leukaemias among 99 animals whereas no tumours were obtained by painting the soles of 14 mice with the same carcinogen (Stein-Werblowsky, unpublished data ; Twort and Twort, 1936).

Kennaway (1955) has compared the induction of human cervical cancer to the production of cancer in animals—what he called the " mouse painting theory ", the carcinogens being possible human carcinogens including smegma (Plaut and Kohn-Speyer, 1947 ; Pratt-Thomas *et al.*, 1956 ; Heins, Dennis and Pratt-Thomas, 1958). Another potential human carcinogen, to our knowledge not yet investigated, may be the human ejaculate. The prostatic and testicular secretions contained therein may have carcinogenic properties analogous to the carcinogenic effects of ovarian extracts. Testosterone for example has been found to induce tumours in Laboratory animals (Horning, 1958). Exposure of basal cells to such exogenous carcinogens is possible during the immediate post-menstrual period when the genital epithelium is of low type, analogous to that of animals in the dioestrus phase.

A number of authors hold that ethnic groups who observe religious abstention in the immediate-post-menstrual phase with or without concomitant circumcision of the male—show a low incidence of cervical cancer (Sorsby, 1931 ; Symeonidis 1951 ; Wynder *et al.*, 1954 ; Gault, 1955 ; Kennaway, 1955 ; Ober and Reiner, 1955 ; Dujovich and Gruliges, 1956). According to Gagnon (1955), total abstention goes together with non-existence of cancer of the cervix. In his series of 13,000 nuns no cancer was found at that site. Towne (1955), on the other hand, reports 6 out of 13,083 cases of cervical cancer in nuns. Hochmann and Ratzkowski (1955) and Casper (1955) confirm the low incidence of cancer of this

type among Jewesses but do not attribute it to the laws of religious abstention, as the majority are no longer aware of their existence.

In view of the diversity of opinions new and more detailed inquiries appear to be called for. Information should be sought regarding periods of abstention within the monthly cycle, irrespective of ethnic and/or religious factors. A relation between such anamnestic data and the experimental findings of this paper might then be established.

SUMMARY

Benzopyrene was painted on the cervix uteri of a group of rats in the dioestrus and oestrus phases respectively. In the dioestrus group 5 malignant and 14 benign lesions were obtained in 53 animals whereas in the oestrus group of 54 animals only 4 benign lesions developed. The possible relevance of these experimental findings to the aetiology of human cervical cancer is discussed.

The author is indebted to Dr. S. S. Epstein for his help and advice in the preparation of the manuscript. Grateful thanks are also due to Dr. C. P. Cherry for several histological diagnoses, to Mrs. P. David for skilful technical assistance and to Mr. E. Savill for the photomicrographs.

REFERENCES

Berenblum, I., Haran-Ghera, N. and Trainin, N.—(1958) *Brit. J. Cancer*, **12**, 402.
Breedis, C.—(1955) *Proc. Amer. Ass. Cancer Res.*, **2**, 7.
Casper, J.—(1955) *Schweiz. Z. Path.*, **18**, 769.—(1958) Abstract, *7th int. Cancer Congr., Lond.* p. 281.
Dujovich, A. and Gruliges, S.—(1956) *Excerpta med., Amst. (Cancer)*. Vol. 4. Abstract 699.
Gagnon, F.—(1955) *Schweiz. Z. Path.*, **18**, 755.
Gardner, W. O., Allen, E., Smith, G. M. and Strong, L. C.—(1938) *J. Amer. med. Ass.*, **110**, 1182.
Gault, E. W.—(1955) *Schweiz. Z. Path.*, **18**, 732.
Glucksmann, A.—(1945) *Cancer Res.*, **5**, 385.
Idem and Cherry, C. P.—(1958) *Brit. J. Cancer*, **12**, 32.
Guimaraes, U. P. and Braz, T.—(1958) Abstract, *7th int. Cancer Congr., Lond.* p. 289.
Hausdorff, H.—(1955) *Zbl. Gynäk.*, **77**, 1854.
von Haam, E. and Scarpelli, D. G.—(1955) *Cancer Res.*, **15**, 449.
Heins, H. C. Jr., Dennis, E. J. and Pratt-Thomas, H. R.—(1958) *Amer. J. Obstet. Gynec.*, **76**, 726.
Hochmann, A., Ratzkowski, E. and Schreiber, H.—(1955) *Brit. J. Cancer*, **9**, 358.
Horning, E. S.—(1958) *Ibid.*, **12**, 414.
Huggins, C.—(1958) Abstract, *7th int. Cancer Congr., Lond.* p. 1.
Kennaway, E. L.—(1955) *Brit. med. J.*, i, 1107.
Koprowska, I., Bogacz, J., Pentikas, C. and Stypulkowski, W.—(1958) *Cancer Res.*, **18**, 1186.
Murphy, E. D.—(1953) *Amer. J. Path.*, **29**, 608.—(1958) Abstract, *7th int. Cancer Congr., Lond.* p. 191.
Ober, W. B. and Reiner, L.—(1955) *Schweiz. Z. Path.*, **18**, 774.
Perry, I. H.—(1936) *Proc. Soc. exp. Biol., N.Y.*, **35**, 325.
Plaut, A. and Kohn-Speyer, A.—(1947) *Science*, **105**, 391.

PRATT-THOMAS, H. R., HEINS, H. C., LATHAM, E., DENNIS, E. J. AND MCIVER, F. A.—(1956) *Cancer*, **9**, 671.
REAGAN, J. W., WENTZ, W. B. AND MACHICAO, N.—(1955) *Arch. Path.*, **60**, 451.
RUNGE, H. AND ZEITZ, H.—(1958) Abstract, *7th int. Cancer Congr., Lond.* p. 290.
SCARPELLI, D. G. AND VON HAAM, E.—(1957) *Amer. J. Path.*, **33**, 1059.
SNELL, G. D.—(1941) ' Biology of the Laboratory Mouse '. Philadelphia (Blakiston), p. 78.
SORSBY, M.—(1931) ' Cancer and Race '. London (Bale & Son).
SYMEONIDIS, A.—(1951) *Acta Un. int. Cancr.*, **7**, 125.
TOWNE, J. E.—(1955) *Amer. J. Obstet. Gynec.*, **69**, 607.
TWORT, J. M. AND TWORT, C. C.—(1936) *J. Path. Bact.*, **42**, 303.
VELLIOS, F. AND GRIFFIN, J.—(1957) *Cancer Res.*, **17**, 365.
WYNDER, E. L., CORNFIELD, J., SCHROFF, P. D. AND DORISWAME, K. R.—(1954) *Amer. J. Obstet. Gynec.*, **68**, 1016.

THE EFFECT OF INCUBATION ON THREE FOWL TUMOUR VIRUSES

H. A. DRAYTON

From the British Empire Cancer Campaign Unit, Agricultural Research Council Poultry Research Centre, West Mains Road, Edinburgh, 9

Received for publication April 22, 1960

THE thermolabile character of chicken sarcoma I filtrates has been noted since 1919 (Rous, Robertson and Oliver, 1919). Later, it was found that incubation at 37° C. for 24 hours resulted in a rapid loss of infectivity even in filtrates of high titre and that inactivation was invariably complete after treatment at 55° C. for 15 minutes. Mueller (1928) first mentioned Gye's finding that this inactivation of Rous I filtrates at moderate temperatures could be prevented for periods up to three days by the addition of HCN.

Gye (1925) ascribed this phenomenon of inactivation to the oxidation of a "specific factor", but later inclined to the view that inactivation was due to "proteolytic ferments in the filtrates acting on a non-living protein specific factor" (Gye and Purdy, 1930). In the light of Oppenheimer's finding that substances like HCN although powerful anti-oxidants, had little effect on proteases (Oppenheimer, 1925), Mueller (1928) adhered to Gye's earlier theory that inactivation was a result of oxidation of a specific component of the virus.

Pirie and Holmes (1931) argued that since incubation at 37° C. even under anaerobic conditions resulted in a loss of potency of Rous I filtrates, atmospheric oxygen need play no part in any oxidation which is assumed to accompany Rous I virus inactivation. They suggested therefore that some oxidiser-catalyst system was closely attached to the virus itself, while admitting that it had not been possible to separate it from the virus nor to identify it chemically.

With the exception of Rous I there is no information on the effect of incubation on the avian tumour viruses. It was thought that a comparative study on the effect of incubation at 37° C. for varying periods of time on the "infectivity" of partially purified Rous I, MH_2 (Murray and Begg, 1930), and PRC_4 (Carr and Campbell, 1958) virus preparations might be of interest, if an attempt were made to correlate any effect on potency with virus material liberated and detected by u.v. spectrophotometric analysis.

MATERIALS AND METHODS

Preparation of normal cell particulate suspensions and of virus suspensions

Suspensions of cell particulates from normal fowl tissues (liver and spleen), from a non-filterable fowl sarcoma, GRCH 16 (Peacock and Peacock, 1953) and partially purified virus suspensions from Rous I, MH_2 and PRC_4 tumours, were prepared by the method originally devised by Carr and Harris (1951) and modified by Bather (1953). In each series of experiments the same initial weight of tissue was used as starting material for the preparation of each of the five suspensions.

Bioassay

Chickens from the highly susceptible inbred Brown Leghorn line maintained at the Poultry Research Centre were used for the bioassay of the three virus preparations before and after treatment.

These birds have been selected for susceptibility to Rous I sarcoma cells and virus, and allow infectivity titrations to be made in young chicks to a constant end-point (Carr and Harris, 1951). The method of Parker and Rivers (1936) was used to estimate the second figure in the end-point dilution. Groups of 4 chicks were used for each serial ten-fold dilution of virus.

The initial untreated virus preparation was made in each case by resuspending the " pellet " obtained after the final high-speed centrifugation in as many ml. of McIlvaine's phosphate-citric buffer (pH 7·2) as there were grams of " wet tumour ", so that the concentration of virus before titration was such that 1 ml. = 1 mg. tumour. " Infectivity " was always recorded as m.i.d./g. of tumour (Carr and Harris, 1951).

EXPERIMENTS

In each experimental series the " infectivity " of the three partially purified virus suspensions was estimated by the bioassay method outlined above. The non-infective character of the GRCH and " normal " cell particulate suspensions was confirmed by inoculation of the undiluted preparations into 4 chicks.

Equal volumes of each of the 5 suspensions suitably diluted (10^{-1}) were subjected to the following treatments:

(1) Heated to 100° for 15 minutes.
(2) Incubated at 37° C. for 4 hours.
(3) ,, 37° C. ,, 8 ,,
(4) ,, 37° C. ,, 16 ,,
(5) ,, 37° C. ,, 24 ,,
(6) ,, 37° C. ,, 24 ,, with addition of M/1 KCN.
(7) Maintained at 0° C. for 24 hours.

In each case after treatment, the virus/cell particulate was sedimented by high speed centrifugation—15,000 g. for 55 minutes—and the supernatant pipetted off, diluted appropriately and examined in the u.v. spectrophotometer (Unicam SP 500) at wave-lengths between 2400 Å and 3000 Å. Optical density was plotted against wave-length and the curves compared. The residue from each treatment, in the case of the three viruses, was resuspended in McIlvaine's buffer pH 7·2 and tested for potency.

Aseptic technique was observed throughout; tests for the presence of contaminating bacteria using nutrient agar slopes incubated at 37° C., were negative.

The results of many series of experiments including some or all of the 7 treatments described above, were consistent; to avoid repetition only those for the most detailed series are reported.

RESULTS

Infectivity

During the first four hours of incubation at 37° C. there is an appreciable decrease in infectivity of each of the three fowl tumour viruses, Rous I, MH_2 and PRC_4. This loss of potency continues with further incubation until by the end

of 24 hours the virus preparations are completely non-infective. Boiling for 15 minutes also results in a complete loss of activity of the three virus preparations. Most of the infectivity of the three virus preparations is retained for 24 hours after incubation at 37° C. with the addition of KCN or after storage at 0° C. (Tables I, II and III). Table IV summarises the 24 hour data alone from other experiments.

TABLE I.—*Effect of Experimental Treatments on Infectivity of Partially Purified Rous I Virus Preparations*

Treatment	Dilution of sample and number of tumours from 4 inoculations of each dilution					"Infectivity" of sample (m.i.d./g. tumour)
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	
Initially untreated	4/4	4/4	1/4	0/4	0/4	$10^{3.7}$
37° C. for 4 hours	4/4	1/4	0/4	—	—	$10^{1.7}$
37° C. for 8 hours	1/4	0/4	0/4	—	—	$10^{0.7}$
37° C. for 16 hours	1/4	0/4	0/4	—	—	$10^{0.7}$
37° C. for 24 hours	0/4	0/4	0/4	—	—	0
37° C. for 24 hours (+ KCN)	4/4	3/4	0/4	—	—	$10^{2.3}$
100° C. for 15 minutes	0/4	0/4	0/4	—	—	0
0° C. for 24 hours	4/4	3/4	0/4	—	—	$10^{2.3}$

TABLE II.—*Effect of Experimental Treatments on Infectivity of Partially Purified MH_2 Virus Preparation*

Treatment	Dilution of sample and number of tumours from 4 inoculations of each dilution					"Infectivity" of sample (m.i.d./g. tumour)
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	
Initially untreated	4/4	4/4	4/4	4/4	1/4	$10^{4.7}$
37° C. for 4 hours	4/4	4/4	3/4	0/4	0/4	$10^{3.3}$
37° C. for 8 hours	4/4	4/4	2/4	0/4	0/4	$10^{3.0}$
37° C. for 16 hours	4/4	1/4	0/4	0/4	0/4	$10^{1.7}$
37° C. for 24 hours	0/4	—	—	—	—	0
37° C. for 24 hours (+ KCN)	4/4	4/4	4/4	4/4	0/4	$10^{4.5}$
100° C. for 15 minutes	0/4	—	—	—	—	0
0° C. for 24 hours	4/4	4/4	4/4	4/4	1/4	$10^{4.7}$

TABLE III.—*Effect of Experimental Treatments on Infectivity of Partially Purified PRC_4 Virus Preparation*

Treatment	Dilution of sample and number of tumours from 4 inoculations of each dilution					"Infectivity" of sample (m.i.d./g. tumour)
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	
Initially untreated	4/4	4/4	4/4	3/4	0/4	$10^{4.3}$
37° C. for 4 hours	4/4	4/4	2/4	0/4	0/4	$10^{3.0}$
37° C. for 8 hours	4/4	0/4	0/4	0/4	0/4	$10^{1.5}$
37° C. for 16 hours	1/4	0/4	0/4	0/4	0/4	$10^{1.0}$
37° C. for 24 hours	0/4	0/4	—	—	—	0
37° C. for 24 hours (+ KCN)	4/4	4/4	4/4	1/4	—	$10^{3.7}$
100° C. for 15 minutes	0/4	—	—	—	—	0
0° C. for 24 hours	4/4	4/4	4/4	3/4	0/4	$10^{4.3}$

TABLE IV.—*Effect on "Infectivity" of Tumour Virus Suspensions and Controls Incubated at 37° C. for 24 hours, Compared with Optical Density at 2800 Å of Material Liberated in Four Series of Experiments*

Experiment		Rous I	MH_2	PRC_4	Rous I (+ KCN)	Normal cell particulates	GRCH
1	Decrease in infectivity (log units)	2·7	4·7	4·3	0·4	—	—
	Optical density at 2800 Å	0·16	0·26	0·20	0·02	0·1	0·08
2	Decrease in infectivity (log units)	5·3	3·7	2·5	0·6	—	—
	Optical density at 2800 Å	0·35	0·18	0·13	0·04	0·05	0·03
3	Decrease in infectivity (log units)	4·3	3·5	4·0	0·6	—	—
	Optical density at 2800 Å	0·28	0·13	0·22	0·02	0·08	0·05
4	Decrease in infectivity (log units)	3·0	5·5	3·7	0·3	—	—
	Optical density at 2800 Å	0·15	0·34	0·2	0·04	0·1	0·05

If we assume an initial infectivity of 100 per cent for the untreated virus preparations, which always fell to zero after 24 hours' incubation at 37° C., then the percentage infectivity at 4, 8 and 16 hours can be estimated from the biossay results, and survival curves deduced. On the basis of such curves it is possible to compare the rates of death of Rous I, MH_2 and PRC_4 viruses under the conditions of these experiments. In this way we obtain a half-life for Rous I virus of approximately 324 minutes (compared with 155 minutes found by Rubin in his experiments (Rubin, 1955)) for MH_2 of 12 hours and 6·3 hours for PRC_4 (Fig. 3).

Ultra-violet absorption

The significant section of the u.v. spectrum examined from 2400–3000 Å is that represented in Fig. 1 and 2, i.e. between 2650 Å and 2900 Å. Each of the three virus preparations incubated at 37° C. yields material which absorbs strongly in the region 2750–2800 Å, and is very likely protein. The amount of protein-like material released increases as the time of incubation proceeds from 4–24 hours (Fig. 2). GRCH 16 preparations and suspensions of normal cell particulates incubated at 37° C. for precisely the same periods as the virus suspensions, do not release material with any detectable u.v. absorption peak in the range examined (Fig. 1).

If the amount of protein-like material (as estimated by spectral absorption maxima) released by each of the three viruses is plotted over the time range 4–24 hours, the curves parallel those showing percentage decrease of infectivity with time (Fig. 3).

KCN successfully inhibits the shedding of protein-like material from all three virus preparations incubated at 37° C. for 24 hours (Fig. 1).

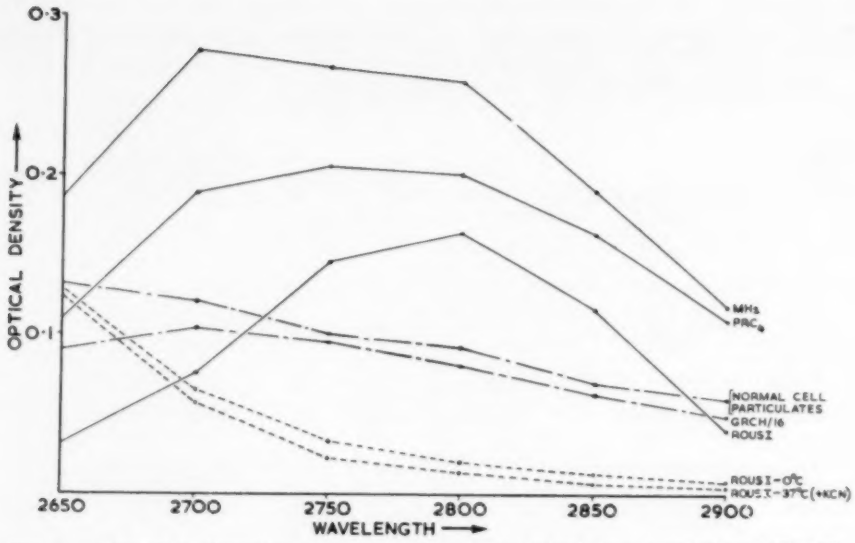


FIG. 1.—Ultra-violet absorption spectra of supernatants derived from preparations indicated after incubation at 37° C. for 24 hours.

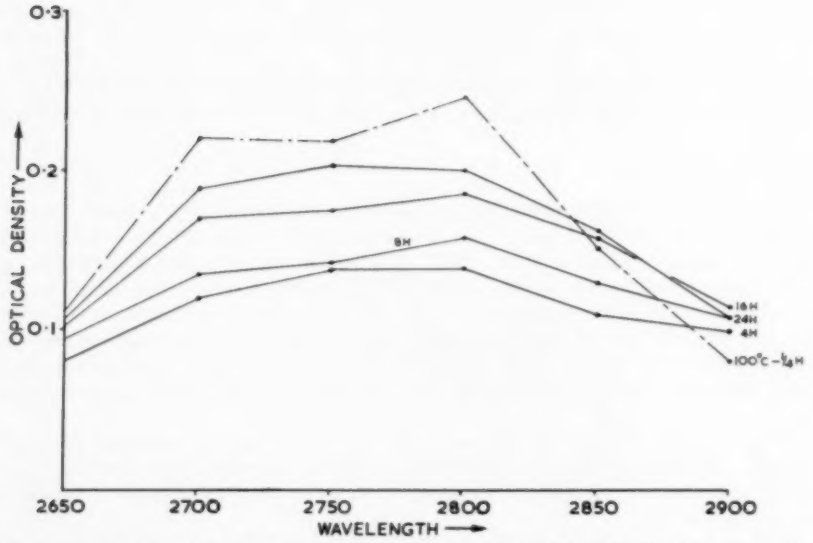


FIG. 2.—Ultra-violet absorption spectra of supernatants derived from PRC_4 virus preparation after incubation at 37° C. for the times indicated.

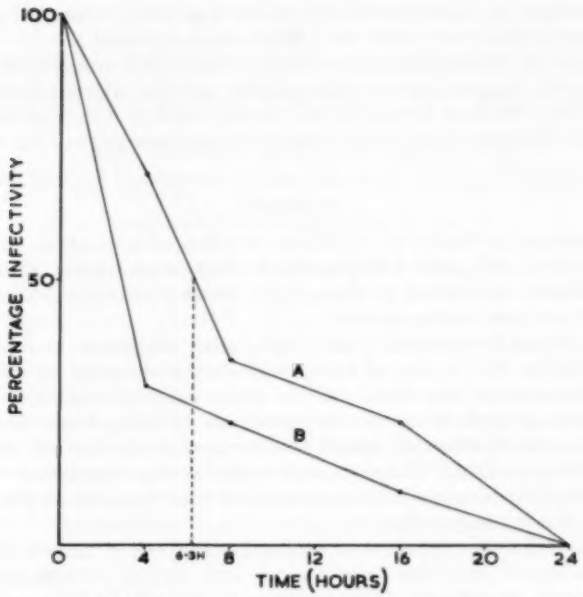


FIG. 3.—Survival curves for PRC_4 virus incubated at 37° C. for periods up to 24 hours.

(A) From infectivity results.
(B) From absorption spectral maxima.

DISCUSSION

The results of these experiments indicate that the decrease in infectivity of partially purified Rous I, MH_2 and PRC_4 virus preparations incubated at 37° C. for a period of 4–24 hours is accompanied by the liberation of increasing amounts of protein-like material in the supernatants after sedimentation. Normal cell particulate and non-virus tumour—GRCH 16—preparations treated in the same manner do not yield material with any detectable absorption peak in the range examined. We can conclude therefore that the protein-like material released in these experiments was derived from the virus particles and not from the host cell contaminants present in some degree in all fowl tumour virus preparations.

This gives confirmation to the theory that the inactivation which ensues after incubation of Rous I, as with MH_2 and PRC_4 virus preparations incubated at 37° C. for periods up to 24 hours results from actual decomposition of virus protein and not merely by simple oxidation which would alter the molecular configuration but would in no way affect its integrity. It is unlikely, however, in view of the u.v. absorption spectra of the material liberated that proteases are implicated in the breakdown as suggested by Gye (Gye and Purdy, 1930).

The decomposition of virus protein is known to be reduced by traces of biological materials (Adams, 1948) and quite possibly from the protein-like material liberated in these experiments. It is therefore very sensitive to in-

significant changes in experimental procedure (e.g. total volume of fluid) and the rates of decomposition may then vary from those reported.

The absence of detectable nucleic acid in the supernatants after any of the treatments would suggest that the protein-like material liberated during inactivation, is not that which is bound to ribonucleic acid in the nucleoids of Rous I virus particles (Epstein, 1958), but is derived from some part of the external coat.

SUMMARY

1. Experiments are described in which the effect of incubation on the biological activity of Rous I, MH_2 and PRC_4 purified virus preparations, as determined by bioassay methods, is studied in relation to virus constituents liberated, as determined by u.v. spectrophotometry.

2. Infectivity of Rous I, MH_2 and PRC_4 virus preparations decreased to zero after incubation at 37° C. for 24 hours and after boiling for 15 minutes.

3. The decrease in infectivity of the three virus preparations incubated at 37° C. for various periods up to 24 hours was paralleled by the liberation of increasing amounts of material, which absorbs strongly in the u.v. at 2750–2800 Å and is very likely protein. Since no such material was detected in GRCH 16 and normal cell particulate controls, it is concluded that the protein-like material was derived from the virus particles.

4. The loss of infectivity which ensues on incubation of Rous I, MH_2 and PRC_4 virus preparations is associated not merely with simple oxidation but by actual decomposition of constituent virus protein.

All expenses in connection with this work were borne by the British Empire Cancer Campaign.

REFERENCES

Adams, M. H.—(1948) *J. gen. Physiol.*, **31**, 417.
Bather, R.—(1953) *Brit. J. Cancer*, **7**, 492.
Carr, J. G. and Campbell, J. G.—(1958) *Ibid.*, **12**, 631.
Idem and Harris, R. J. C.—(1951) *Ibid.*, **5**, 83.
Epstein, M. A.—(1958) *Nature Lond.*, **181**, 1808.
Gye, W. E.—(1925) *Lancet*, ii, 109.
Idem and Purdy, W. J.—(1930) *Brit. J. exp. Path.*, **11**, 282.
Mueller, J. H.—(1928) *J. exp. Med.*, **48**, 343.
Murray, J. A. and Begg, A. M.—(1930) *Sci. Rep. Imp. Cancer Res. Fd. Lond.*, **9**, 1.
Oppenheimer, C.—(1925) 'Die Fermente und ihre wirKungen'. Leipsic (Georg Thieme), 5th ed., Vol. I, p. 75.
Parker, R. F. and Rivers, T. M.—(1936) *J. exp. Med.*, **64**, 439.
Peacock, P. R. and Peacock, A.—(1953) *Brit. J. Cancer*, **7**, 120.
Pirie, A. and Holmes, B. E.—(1931) *Brit. J. exp. Path.*, **12**, 127.
Rous, P., Robertson, H. and Oliver, J.—(1919) *J. exp. Med.*, **29**, 308.
Rubin, H.—(1955) *Virology*, **1**, 445.

ABNORMAL DISTRIBUTION OF NUCLEIC ACID IN TISSUE CULTURE CELLS INFECTED WITH POLYOMA VIRUS

A. C. ALLISON AND J. A. ARMSTRONG

From the National Institute for Medical Research, Mill Hill, London, N.W.7

Received for publication April 13, 1960

THE polyoma virus, originally isolated from mice, has been shown to produce multiple tumours when inoculated into new-born mice and hamsters (Stewart, Eddy and Borgese, 1958). Growth of the virus in mouse embryo tissue cultures is accompanied by a regular cytopathic effect, notably clumping of cells and detachment from glass surfaces (Eddy, Stewart and Berkeley, 1958). The fluorescent antibody technique has shown that in polyoma-infected tissue cultures of mouse embryo cells viral antigenic material develops first in the host cell nuclei and appears later in the cytoplasm (Henle, Deinhart and Rodriguez, 1959). Electron micrographs of ultrathin sections of tissue culture cells infected with the prototype SE and Mill Hill strains of polyoma virus have shown large numbers of virus-like particles, mostly in the nuclei (Bernhard, Febvre and Cramer, 1959; Dmochowski, Grey and Magee, 1959; Negroni, Dourmashkin and Chesterman, 1959).

Evidence concerning the nucleic acid component of polyoma virus has been conflicting. Cytochemical changes in infected mouse lymphoma cells were reported by Love and Rabson (1959) who used the toluidine blue–molybdate and Feulgen techniques. Cellular enlargement was associated with the appearance of abnormal nuclear vacuoles which developed a staining reaction for ribonucleoprotein. From these findings, it was inferred that normal nuclear mechanisms of ribonucleoprotein synthesis may be diverted into the process of virus replication. No evidence of an increased synthesis of deoxyribonucleic acid (DNA) was found. On the other hand, Di Mayorca *et al.* (1959) have very recently reported that virus-free nucleic acid extracts of polyoma-infected tissue cultures could reproduce the typical infection in other tissue cultures. The infectivity of the extracts, unlike that of intact virus, was abolished by incubation with deoxyribonuclease. Ribonuclease had no effect, and it was concluded that polyoma is probably a DNA virus.

In view of this discrepancy it may be helpful to record briefly some observations recently made in this laboratory which are at variance with the previously published cytochemical data. The technique employed has been the acridine orange polychromatic fluorescence technique for differentiation of nucleic acids, which is particularly suited to the study of virus cytopathology in tissue cultures (Armstrong, 1956; Armstrong and Niven, 1957).

MATERIALS AND METHODS

Cells

Three cell types were used. New-born mouse kidney and mouse embryo cell cultures were prepared as described by Rowe *et al.* (1959). They were grown for four days as stationary cultures in flat 4-ounce bottles in the following medium :

Gey's saline, 80 parts ; lactalbumin hydrolysate (5 per cent of stock) 10 parts ; calf serum, 10 parts ; penicillin 100 units per ml. ; streptomycin 100 μg. per ml. ; and Mycostatin 50 units per ml. The cells were trypsinized, washed and transferred in 2 ml. volumes containing 10^6 cells into test-tubes with coverslips. After 3 days' growth in the same nutrient medium, the medium was replaced with another containing 5 per cent calf serum and the virus inoculum. A strain of "L" cells (obtained from Dr. J. C. N. Westwood, Microbiological Research Establishment, Porton, Wilts.) was maintained in a medium consisting of Gey's saline 70 parts ; calf serum, 20 parts ; yeast extract (1 per cent of stock) 10 parts ; lactalbumin hydrolysate (5 per cent of stock) 10 parts ; and antibiotics as above. Trypsinized, washed cells were transferred to test tubes containing coverslips, grown and infected in a medium containing 10 parts of calf serum.

Virus

SE polyoma virus, batch 3E29–4, obtained from Dr. B. E. Eddy, National Cancer Institute, Bethesda, Md., U.S.A., was propagated in mouse embryo cell cultures in flat bottles. After 7 days' incubation with two changes of medium the cells were removed and treated with fluorocarbon (Giardi, 1959) to prepare stock virus. Virus was titrated by haemagglutination of guinea-pig cells and infectivity in mouse enbryo tissue cultures (Rowe *et al.*, 1959).

Microscopy

Coverslips from control and infected cultures were removed at daily intervals up to 8 days after virus inoculation, rinsed in tris-buffered Gey's solution (pH 7·4) and fixed in absolute alcohol. The method of staining coverslip cultures with 0·05 per cent acridine orange (C.I. 788) in acetate-HCl buffer (pH 2·7) was described by Armstrong and Hopper (1959). Examination was by dark-field fluorescence microscopy, using the blue-violet emission of a high pressure mercury vapour lamp for activation of fluorescence. Other cultures were fixed for 20 minutes in 1 per cent osmium tetroxide buffered to pH 7·4 (Palade, 1952) ; after rinsing in Gey's solution they were allowed to stand overnight in 70 per cent ethanol and finally mounted in water for examination by phase-contrast microscopy.

OBSERVATIONS

Virus yield

The appearance of infective virus in mouse kidney cells and culture fluid after inoculation with 10^5 TCD_{50} of polyoma virus is shown in Fig. 1. Very little virus could be recovered at 24 hours. There was a considerable increase of cell-associated virus between the second and third days after infection, with very little virus liberation into the culture medium. From the third to the eighth day after infection there was a slower increase in cell-associated virus and some increase in the virus liberated into the medium. The latter, however, was never more than one-tenth of the total virus in the preparation. In cultures of "L" cells virus yield was low at all times and only a small proportion of cells showed morphological changes.

Cytopathic effect

Unstained cultures of the mouse kidney and mouse embryo cells examined under the low power of the microscope showed no change until the third day

after infection, when some cells appeared to be clumped, rounded and refractile. This appearance increased daily, with progressive detachment of cells into the culture medium. But on the eighth day after infection, when disintegration of the cell sheet was sufficient to be visible to the naked eye, large intervening areas containing apparently normal cells were always still present. The cultures of "L" cells showed less marked but consistent cytopathic effects between the fifth and eighth days after infection.

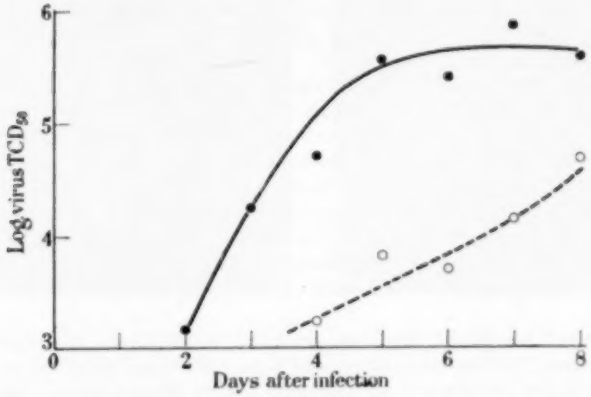


FIG. 1.—Appearance of cell-associated virus (continuous line) and virus in medium (interrupted line) in mouse kidney cultures infected with polyoma virus.

Cytology

The fluorescence of cells in uninfected mouse kidney cell cultures after treatment with acridine orange is reproduced in black and white in Fig. 2. In actual specimens the nuclear margin and chromatin network (including nucleolus-associated chromatin) emitted the greenish-yellow fluorescence which is typical of DNA-containing elements. This contrasted sharply with the orange-red colour of the nucleoli themselves, and of the ribonucleoprotein which is scattered throughout the cytoplasm.

No distinctive cytological changes were observed in these cultures up to 3 days after inoculation with polyoma virus, but on the fourth day there were multiple foci in which cells had become rounded and were easily detached from the glass surface. From this time up to the eighth day of infection an unusual type of intranuclear structure was detectable in many of the cells close to where rounding up and cell detachment were in progress. It was found only occasionally in cells of the intervening, and intact, parts of the cell sheet. The material concerned gave an intense greenish-yellow fluorescence of the DNA type. In 4 day-infected cultures it was mostly located in one or more discrete centres within the nucleoplasm ; but by the eighth day a high percentage of the affected nuclei were almost completely filled. Detection of the earliest stage was made difficult by the presence of rather large chromatin granules in the normal cell nuclei, this being a constant feature of many uninfected murine cells in tissue culture (Fig. 2) ; some nuclei showing minimal alteration of the DNA pattern after infection may

have been overlooked on this account. Nuclear fragmentation was rarely seen in spite of progressive accumulation of the abnormal intranuclear material. As illustrated in Fig. 3–6, the appearances were very different from those of a non-specific degeneration or of pyknosis. The figures show four typically affected nuclei arranged in order of increasing abnormality; the black and white prints were prepared from original colour transparencies. The positions of nucleoli, identifiable because of the red fluorescence of their RNA, are indicated as a guide in Fig. 2 and 4; the remaining nuclear fluorescence was entirely of the DNA type. In addition to nuclear changes there was vacuolation of the cytoplasm in some of these cells (Fig. 3 and 6). No regular change was noted in the structure of the nucleoli or in their fluorescent intensity. None of the infected mouse kidney cell cultures showed evidence of DNA-containing material in the cytoplasm.

Almost identical changes were found in the polyoma-infected mouse embryo cultures; and a few scattered cells with the same abnormal patterns of intranuclear DNA were present, in the corresponding cultures of "L" strain cells. In the latter, however, occasional cells with abnormal nuclei had in their cytoplasm a prominent paranuclear mass of greenish-yellow fluorescent material (Fig. 7). This was quite distinct from the dull green fluorescence sometimes found in a hypertrophied Golgi zone of cultured epithelial cells. It was also the only indication of a cytoplasmic DNA reaction in the polyoma-infected cultures at our disposal.

Phase-contrast study of the osmium-fixed kidney cell and mouse embryo cultures confirmed that nuclear changes of a peculiar kind occurred in the vicinity of the focal cytopathic effect. It has been shown that osmium fixation avoids structural artefacts which are sometimes brought about by the more commonly used histological fixatives (Strangeways and Canti, 1927). Moreover, cytological details may be revealed by phase-contrast with greater clarity than is usually possible in the equivalent living cell. A normal cell nucleus is shown in Fig. 8; the nucleoli are very dense relative to the surrounding nucleoplasm, which is faintly stippled. The type of nucleus seen in Fig. 9, however, occurred only after the fourth day of infection with polyoma virus. Irregular patches of somewhat lower density than the nucleoli, and of varying size, are present in the nucleoplasm; their appearance resembles closely that of the material revealed by fluorescence microscopy after alcohol fixation (Fig. 3 and 4). In view of this it seems likely that the distribution of nuclear contents shown by the fluorescence microscope was not, in this case, much influenced by fixation artefact.

EXPLANATION OF PLATES

FIG. 2–7 are fluorescence photomicrographs of cells in tissue culture, stained by the acridine orange technique. Prints were made from colour transparencies. The nucleoli (shown by arrows in Fig. 1 and 3) were red (RNA), the remaining nuclear fluorescence greenish-yellow (DNA).

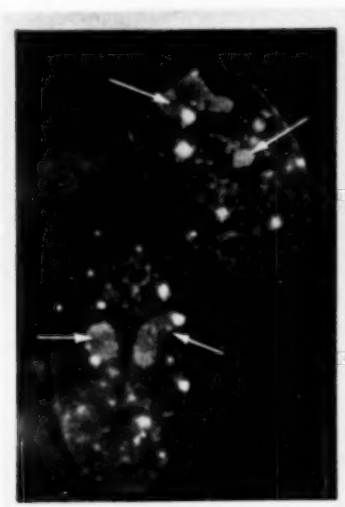
FIG. 2.—Uninfected mouse kidney cells, × 1200.

FIG. 3–6.—Abnormal patterns of nuclear DNA fluorescence in mouse kidney cells, between 4 and 8 days after infection with polyoma virus, ×1200.

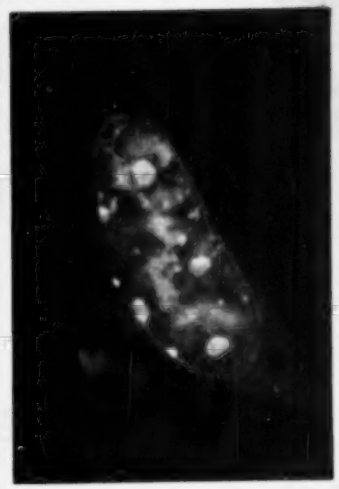
FIG. 7.—"L" strain cell showing a cytoplasmic mass of DNA-type fluorescence 6 days after infection with polyoma virus, ×1700.

FIG. 8.—Phase-contrast photomicrograph, showing the nucleus of a normal mouse kidney cell in tissue culture. OsO_4 fixation, ×1800.

FIG. 9.—Similar preparation to Fig. 8, but from a culture infected 8 days previously with polyoma virus. OsO_4 fixation, ×1800.



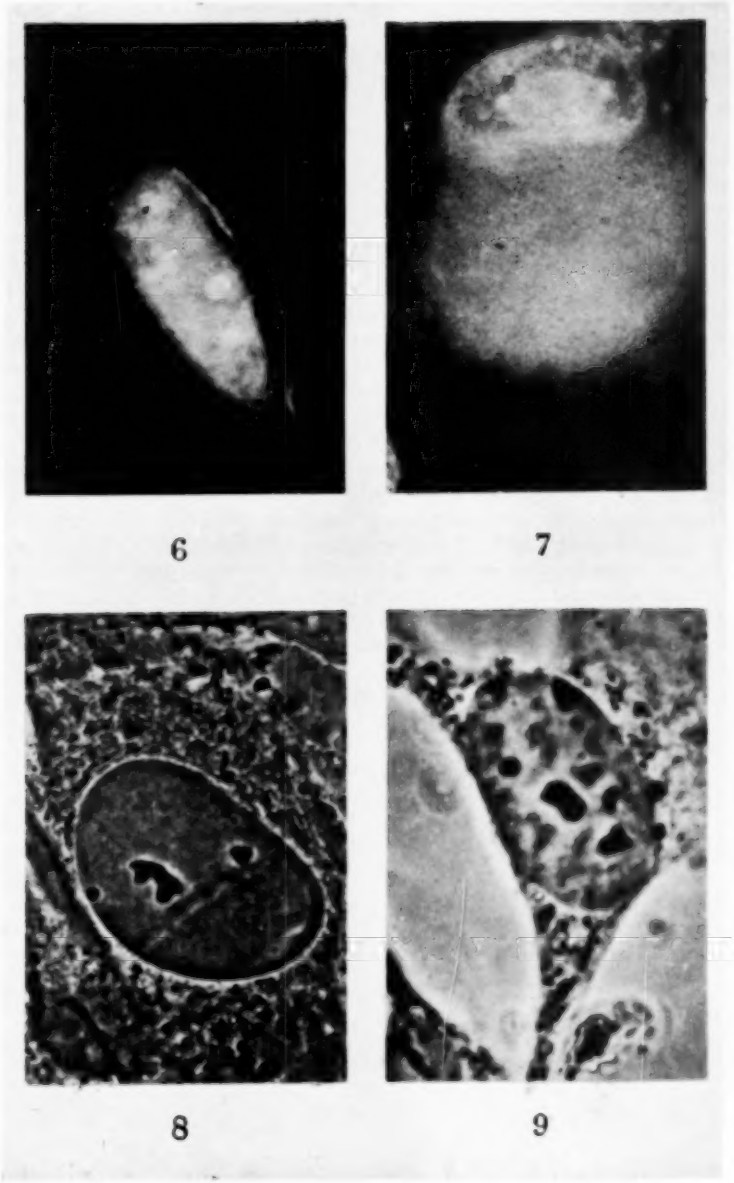
2



3

4

5



6 7

8 9

Allison and Armstrong.

COMMENT

The cytochemical changes reported here are reminiscent of the abnormal patterns of intranuclear DNA which are found in human cell cultures infected with adenoviruses, and which are clearly demonstrated by the acridine orange technique (Armstrong and Hopper, 1959). However, with polyoma the DNA changes were not associated with the remarkable distortion of nuclear morphology which is so typical of adenovirus infections and which is greatly influenced by alcohol fixation. The present findings would appear, therefore, to be consistent with the replication of a DNA virus within the nuclei of susceptible murine cells. Close inspection of the control specimens revealed no comparable DNA pattern, so that the possibility of a spontaneous development of bizarre nuclear forms is very unlikely. We interpret the present findings in polyoma-infected cultures as additional evidence for the view, expressed by Di Mayorca *et al.* (1959), that the polyoma agent is in all probability a DNA virus.

The first indication of infection is the appearance of multiple foci of DNA-containing material in the nucleoplasm. The findings suggest that these enlarge, coalesce and eventually fill the nucleus. Cells at this stage of virus development presumably correspond to those which have shown large numbers of virus-like inclusions when examined by electron microscopy. In infected " L " cell cultures, a few cells were seen with DNA-containing material in the cytoplasm, suggesting a discharge of virus nucleic acid from the nucleus into the cytoplasm, but in the mouse kidney and mouse embryo cells there were no signs of cytoplasmic DNA. This might be related to the fact that moribund cells with large amounts of intranuclear virus are detached from the coverslips before disintegration and release of virus. Certainly the occurrence of recognizable cells in the medium coincided with the appearance in it of detectable virus. In general, the fluorescence induced by acridine orange staining is comparable with that obtained in the earlier stages of infection by means of the fluorescent antibody technique (Henle *et al.*, 1959), but with the latter some cells in the late cultures showed cytoplasmic fluorescence. This might be due to the passage of virus from the nucleus into the cytoplasm, but alternatively could indicate the presence in the cytoplasm of antigen not accompanied by nucleic acid and not therefore visualized by acridine orange.

A relatively long latent period and slow progression of infection with the polyoma virus appears to be the rule, and despite the use of relatively large virus inocula only a minority of cells showed cytopathic effects and accumulation of DNA-containing material in the nuclei. It is conceivable that other parts of the cell sheet were infected but transformed into tumour-like cells releasing very little virus as suggested recently by Vogt and Dulbecco (1960).

SUMMARY

The progress of infection with polyoma virus in tissue cultures of mouse kidney cells, mouse embryo cells and " L " cells has been followed by titration of virus and the appearance of cytochemical changes.

Infective virus accumulates gradually and is slowly released from infected cells. In spite of large initial virus inocula only a minority of cells showed characteristic cytopathic effects.

After infection the first change detectable in cells stained with acridine orange and examined by fluorescence microscopy was the appearance of abnormal DNA-containing foci in the nuclei. At a later stage cells became detached from the coverslips and the nuclei of some were by then almost filled with the DNA-containing material. It is concluded that polyoma virus contains DNA and multiplies in the nuclei of susceptible cells.

We are indebted to Mr. M. R. Young for taking the photomicrographs.

REFERENCES

ARMSTRONG, J. A.—(1956) *Exp. Cell. Res.*, **11**, 640.
Idem AND HOPPER, P. K.—(1959) *Ibid.*, **16**, 584.
Idem AND NIVEN, J. S. F.—(1957) *Nature, Lond.*, **180**, 1335.
BERNHARD, W., FEBVRE, H. L. AND CRAMER, R.—(1959) *C.R. Acad. Sci., Paris*, **249**, 483.
DI MAYORCA, G. A., EDDY, B. E., STEWART, S. E., HUNTER, W. S., FRIEND, C. AND BENDICH, A.—(1959) *Proc. nat. Acad. Sci., Wash.*, **45**, 1804.
DMOCHOWSKI, L., GREY, C. E. AND MAGEE, L. A.—(1959) *Proc. Soc. exp. Biol. N.Y.*, **102**, 575.
EDDY, B. E., STEWART, S. E. AND BERKELEY, W.—(1958) *Ibid.*, **98**, 848.
GIARDI, A. J.—(1959) *Virology*, **9**, 488.
HENLE, G., DEINHARDT, F. AND RODRIGUEZ, J.—(1959) *Ibid.*, **8**, 388.
LOVE, R. AND RABSON, A. S.—(1959) *J. nat. Cancer Inst.*, **23**, 875.
NEGRONI, G., DOURMASHKIN, R. AND CHESTERMAN, F. C.—(1959) *Brit. med. J.*, ii, 1359.
PALADE, G. E.—(1952) *J. exp. Med.*, **95**, 285.
ROWE, W. P., HARTLEY, J. W., ESTES, J. D. AND HUEBNER, R. J.—(1959) *Ibid.*, **109**, 379.
STEWART, S. E., EDDY, B. E. AND BORGESE, N. G.—(1958) *J. nat. Cancer Inst.*, **20**, 1223.
STRANGEWAYS, T. S. P. AND CANTI, R. G.—(1927) *Quart. J. micr. Sci.*, **71**, 1.
VOGT, M. AND DULBECCO, R.—(1960) *Proc. nat. Acad. Sci. Wash.*, **46**, 365.

THE EFFECT OF NITROFURAZONE ON THE TESTES AND ACCESSORY SEX ORGANS OF NORMAL RATS AND RATS BEARING THE WALKER CARCINOMA 256

D. G. MONTEMURRO*

From the Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, London, S.W.3

Received for publication March 24, 1960

PROFOUND changes in the endocrinology of the host result from the growth of not only the so-called functional endocrine tumours but also from the progressive growth of non-functional tumours. Begg and Stewart (1952) and Begg (1955) observed atrophy of the testes, seminal vesicle and prostate of rats bearing the Walker carcinoma. This atrophy could not be influenced by force-feeding designed to maintain carcass weight of the tumour-bearer (Begg, 1958). They found, however, that the atrophy could be prevented by the exogenous administration of serum gonadotrophin and suggested that: " . . . the presence of a tumour produces a deficiency of gonadotrophic hormone in the rat". These observations have been confirmed and extended by Haddow, Horning and Carlton Smith (1957), who found that testicular changes were not as consistent as those reported previously and were restricted to an increase in number and lipid content of the Sertoli cells. Marked hypertrophy of the adrenals was a consistent finding, and in some cases was associated with hypertrophic changes in the pituitary gland. Atrophy of the accessory sex organs could be reversed by testosterone propionate, serum gonadotrophin or by the subcutaneous implantation of pituitary glands.

Nissim (1957) found that nitrofurazone, an anti-bacterial drug sometimes used in infections of the urinary tract, when added to the diet of mice in concentrations of 0·15–0·3 per cent resulted in atrophy of the spermatogenic epithelium of the testes, interstitial cell hyperplasia, and hypertrophy of the seminal vesicle. The effect was absent in hypophysectomized mice, and he suggested that the androgenic action may be a manifestation of hyperactivity of the pituitary due to the release from a pituitary inhibiting factor normally liberated from the germ cells of the testes. Castration cells in the pituitary—an indication of hyperactivity of gonadotrophic basophils—have been reported by Nelson and Steinberger (1952) following the administration of furadroxyl, a drug having a similar action to that of nitrofurazone. The present work deals with some effects of nitrofurazone in normal and tumour-bearing rats. Should nitrofurazone be shown to have an androgenic action in the rat as it does in the mouse, it would then be possible to employ the drug as a stimulus to endogenous pituitary gonadotrophin secretion in tumour-bearing rats and prevent progressive atrophy of the accessory sex organs.

* British Empire Cancer Campaign Exchange Fellow, 1958–60.

MATERIALS AND METHODS

Male albino rats from the colony at the Chester Beatty Research Institute were used throughout. They were 11 weeks old at the beginning of the experiment and weighed roughly 300 g. The animals were housed six to a cage and fed a semi-synthetic diet and water *ad libitum*. The composition of the basic diet used in these experiments is shown in Table I. Nitrofurazone was added to the dry diet in concentrations of 0·15 and 0·30 per cent, which represent the concentrations found by Nissim (1957) to produce seminal vesicle and prostatic hypertrophy in mice.

TABLE I.—*Composition of the Diet*

Constituent	Per cent by weight
Wheat flour	68·6
Casein	11·5
Milk powder	8·0
Margarine	3·3
Bemax	2·5
Yeast	2·4
Cod liver oil	1·6
Calcium carbonate	1·3
Glaxo salt mixture	0·8

The dry diet was mixed with sufficient water to make a dough-like paste which was then fed to the animals *ad libitum*.

The Walker carcinoma 256 serially transplanted at this Institute, grows very rapidly and within 14 days may weigh in excess of 70 g. Accordingly, all animals were sacrificed by an overdose of ether anaesthesia 13 days after the subcutaneous implantation of the tumour. At necropsy the testes, prostate, seminal vesicle and tumour were weighed to the nearest 0·01 g. The seminal vesicle was stripped of coagulating gland and ligated at its junction with the ductus deferens before excision to prevent loss of seminal fluid. All tissues were fixed in Bouin's solution, embedded in paraffin, sectioned at 5 microns and stained with haematoxylin and eosin.

RESULTS

(a) *The effect of nitrofurazone feeding on the testes and accessory sex organs of normal male rats*

Eighteen rats of the same age and approximate weight were used for this experiment. Six were fed the normal basic diet and acted as controls; the remaining 12 were divided into two groups receiving the basic diet supplemented with nitrofurazone 0·15 per cent in one, and 0·50 per cent in the other. The latter dose had to be reduced to 0·30 per cent after three days due to the death of one rat and general physiological deterioration of the remaining five. After 25 days the experiment was terminated. The weights of the testes, seminal vesicle and prostate are shown in Table II. Nitrofurazone feeding resulted in an appreciable loss of body weight in both groups of experimental animals; the greater weight loss was observed in those rats receiving the larger dose of the drug. Although food consumptions were not recorded, daily observation of the animals gave the impression that the inhibition of food intake was proportional to the

dose of nitrofurazone. In order to obviate the effects of the accompanying weight loss, the weights of the organs shown in Table II are expressed as a percentage of of the body weight at death. Testicular atrophy was evident in all rats receiving the drug but was greater in those fed the smaller dose of the drug. Histologically, the testes showed profound degeneration of the spermatogenic epithelium (Fig. 1*a*). Many tubules were filled with a faintly acidophilic, acellular, colloid or mucoid substance. The same substance filled much of the interstitial spaces normally occupied by Leydig tissue. However, hyperplasia of the interstitial cells as described by Nissim (1957) in the mouse was not evident.

TABLE II.—*The Effect of Nitrofurazone Feeding on Body Weight, Testes and Accessory Sex Organs of Normal Male Rats*

	Number	Death weight (g.)	Testes	Seminal vesicle	Prostate
			(mg./100 g. body weight)		
Normal diet	6	522 ± 21*	726 ± 28	209 ± 20	105 ± 9
0·15% nitrofurazone in diet	6	366 ± 10 (<0·001)	377 ± 27 (<0·001)	349 ± 48 (<0·02)	120 ± 10
0·30% nitrofurazone in diet	5	307 ± 13 (<0·001)	514 ± 52 (<0·01)	224 ± 30	94 ± 38

* Mean ± standard error.
( ) Statistical probability of difference from normal diet.

The mean absolute weight of the seminal vesicles in the group of rats fed the lower dose of nitrofurazone was greater than that of the rats on the basic diet. When expressed as a percentage of the body weight this increase (140 mg.) was statistically significant at the $P < 0{\cdot}02$ level. The increase in proportionate weight of the organ in the group of rats fed the higher dose of nitrofurazone was less obvious. Histologically, the seminal vesicles of the experimental animals did not differ from those of the normal rats. The weights and histology of the prostates of the experimental animals were not unlike those of the controls.

(b) *The effect of nitrofurazone feeding on the testes and accessory sex organs of rats bearing the Walker carcinoma* 256

Thirty 11-week-old rats of approximately the same weight received subcutaneous implants of the Walker carcinoma 256. Eighteen were given the basic diet, and 12 the basic diet supplemented with 0·15 per cent nitrofurazone *ad libitum*. Twelve normal rats of the same age and weight fed the basic diet alone, served as controls. Thirteen days after the implant all animals were sacrificed by an overdose of ether anaesthesia. The weights of the tumours testes and accessory sex organs at necropsy are shown in Table III. Once again, the mean weights are expressed as a percentage of the final body weight. Growth of the Walker tumour had no notable effect on the gross weight of the testes when compared with that of the non-tumour-bearing controls. However, atrophy of the accessory sex organs of the tumour-bearers was a consistent finding. The mean proportionate weight of the seminal vesicle was 44 per cent, and that of the prostate 46 per cent lower than those of the normal rats. Haddow, Horning and Carlton Smith (1957) have shown this atrophy to be roughly proportional to the weight of the tumour. Fig. 2 shows the relationship between seminal vesicle

TABLE III.—*The Effect of Nitrofurazone on the Testes and Accessory Sex Organs of Tumour-bearing Male Rats*

	Number	Final body weight (g.)	Tumour weight (% body weight)	Testes (mg./100 g. body weight)	Seminal vesicle (mg./100 g. body weight)	Prostate (mg./100 g. body weight)
Normal rats (basic diet)	12	492±14*	—	786±23	218±13	122±9
P		<0·1	—	<0·3	<0·01	<0·001
Tumour-bearers (basic diet)	18	413±19	13·8±1·9	844±35	122±20	66±7
P		<0·01	<0·6	<0·001	<0·7	<0·8
Tumour-bearers (0·15% nitrofurazone in diet)	12	328±22	15·2±0·8	461±10	110±14	71±9

* Mean ± standard error.

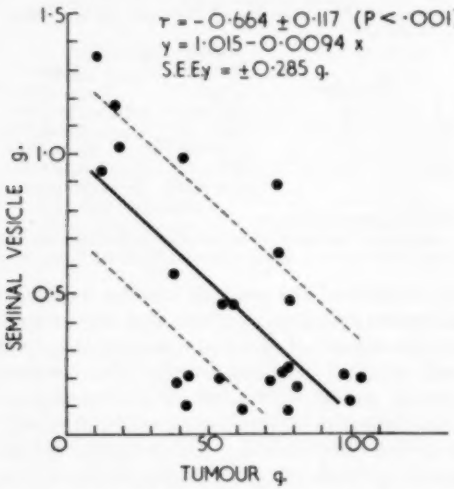


FIG. 2.—Relationship between the weight of the seminal vesicle and the weight of the tumour in 24 rats bearing the Walker carcinoma 256.

weight and tumour weight in 24 untreated tumour-bearers (18 from this experiment and 6 from the experiment described in Section c). Although the scatter is great, a statistically significant negative correlation ($r = -0{\cdot}664 \pm 0{\cdot}117$) could be demonstrated between the two measurements. Roughly 43 per cent of any decrease in seminal vesicle weight is associated with an increase in tumour weight.

The feeding of nitrofurazone to tumour-bearing rats resulted in body weight loss and profound testicular degeneration similar to that observed in normal rats (Section a), but had no effect on the seminal vesicle and prostatic atrophy associated with the growing tumour. The drug had no notable effect on the rate of growth of the tumour.

(c) *The effect of nitrofurazone administered intraperitoneally*

Six tumour-bearing rats were treated daily with 10 mg. of nitrofurazone suspended in 0·4 ml. of arachis oil. Six untreated tumour-bearers and six normal

rats acted as controls. Injections were started on the second day after implantation of the tumour and were continued for 10 days. The results of this experiment are seen in Table IV. As expected, growth of the Walker tumour resulted

TABLE IV.—*The Effect of Nitrofurazone Administered Intraperitoneally on the Testes and Accessory Sex Organs of Tumour-bearing Rats*

	Number	Final body weight (g.)	Tumour weight (% body weight)	Testes (mg./100 g. body weight)	Seminal vesicle	Prostate
Normal rats	6	361±14*	—	1085±41	178±11	92±7
P		<0·2	—	<0·01	<0·001	<0·02
Untreated tumour-bearers	6	403±21	17·4±1·2	845±59	60±14	54±11
P		<0·9	<0·5	<0·9	<0·01	<0·5
Tumour-bearers (10 mg. nitrofurazone daily for 10 days)	6	406±9	14·2±2·0	889±51	124±8	64±4

* Mean ± standard error.

in marked seminal vesicle and prostatic atrophy. The epithelial lining of the seminal vesicle was altered from the high columnar type to a low cuboidal non-secretory type (Fig. 3*a*). There was a decrease in seminal fluid and an increase in the fibromuscular stroma of the gland. Atrophy of the testes was more noticeable than in the previous experiment; however, sections of the testes stained with haematoxylin and eosin revealed no obvious alteration in testicular structure. Haddow, Horning and Carlton Smith (1957) have shown that Sudan IV staining of frozen sections of the testes reveals an increase in number and lipid content of the Sertoli cells in the tumour-bearer, which was taken as evidence of some abnormality in testicular function.

Treatment with 10 mg. of nitrofurazone daily for 10 days had no effect on body weight nor on the weight of the tumour at necropsy when compared with the untreated tumour-bearers. Nor were the testes of the treated group of rats different from those of the untreated tumour-bearers in gross weight and in histological appearance. In fact, the only notable effect of this treatment was the almost complete restoration of the weight and histological appearance of the seminal vesicle to that seen in normal male rats of the same age (Fig. 3*b*). Changes in the weight and histology of the prostate were less obvious. Thus the atrophic changes in the seminal vesicle and to a lesser degree in the prostate, can be largely prevented by the intraperitoneal administration of nitrofurazone.

Larger doses of nitrofurazone (30 mg./day for four days) proved fatal to two out of six tumour-bearers. The remaining four showed, at autopsy, profound testicular atrophy, while the seminal vesicles were slightly but not significantly larger than those of the untreated tumour-bearers. The body weights and tumour weights of these four treated animals did not differ from those of the untreated group.

DISCUSSION

There appears to be a growing belief among clinicians and experimentalists that the progressive growth of tumours has widespread constitutional effects on the host–depression of liver catalase activity, hypertrophy of adrenal and pituitary glands, atrophy of the gonads and accessory sex organs, and ultimately malignant

cachexia, to mention but a few. The results of the present work confirm the previously-made observations of Begg (1955), Begg and Stewart (1952) and of Haddow, Horning and Carlton Smith (1957) that the growth of the Walker rat carcinoma 256 results in atrophic changes in the seminal vesicle and prostate, and to a less notable extent in the testes. Microscopically the accessory sex organs present a picture of grossly diminished or complete absence of secretion, reduction in the height of the cells of the secretory epithelium and an increase in fibro-muscular stroma. These changes can be prevented or reversed by the exogenous administration of serum gonadotrophin (Begg, 1955), testosterone propionate, or by the implantation of fresh pituitary glands (Haddow *et al.*, 1957). The results of the present study show that a protective action against these changes is provided by the daily administration of nitrofurazone (Table IV and Fig. 3*b*).

The androgenic action of nitrofurazone shown by Nissim (1957) to be present in the mouse may also be obtained in the rat. Both species respond to treatment with profound degenerative changes in the spermatogenic epithelium of the seminiferous tubules, but hypertrophy of the accessory sex organs is less dramatic than that described in the mouse. The reason for this difference may be that degeneration of the spermatogenic epithelium in the rat testes was not accompanied by any noticeable hyperplasia of the interstitial tissue. Hyperplasia of Leydig-cell tissue was more apparent than real, due to the large interstitial spaces left by the shrunken and distorted atrophic tubules (Fig. 1*a*). Further, mitotic figures were not more numerous in the interstitial tissue of nitrofurazone-treated rats than in that of untreated normal rats.

The results of these experiments allow some comment on the possible mechanism of the androgenic action of nitrofurazone. Nissim (1957) suggested that this action is attributable to increased pituitary gonadotrophin activity consequent on the withdrawal of an " inhibitory substance " normally liberated by the intact seminiferous tubules. In the experiments on the rat reported here, the maximum hypertrophy of the seminal vesicle—or, more accurately, the greatest inhibition of seminal vesicle atrophy—was seen in those rats that did not show degenerative changes in the spermatogenic epithelium (Table IV). This would indicate that the postulated increase in pituitary gonadotrophin activity following treatment with nitrofurazone is not necessarily dependent on initial degeneration of the spermatogenic epithelium of the testes.

An adequate explanation for the failure of nitrofurazone feeding to prevent atrophy of the accessory sex organs of the rats shown in Table III is wanting However, since administration of the drug by the intraperitoneal route did not produce anorexia and weight loss, the severe under-nutrition associated with the feeding experiments may have resulted in a pituitary gland refractory to any stimulatory action of the drug. Mulinos and Pomerantz (1940) have compared the hormonal imbalances of malnutrition to that of surgical hypophysectomy and described this phenomenon as a dietary pseudo-hypophysectomy.

Some degree of nutritional deficiency or metabolic alteration probably plays a major role on the atrophic changes seen in the accessory sex organs and to a lesser extent in the gonads associated with the progressive growth of a tumour. Hormonal imbalances may be effected by retention of gonadotrophic hormone by the pituitary basophils in a similar manner to that described by Rinaldini (1949) and Pearse and Rinaldini (1950) in semi-starved rats. Experiments to assess the gonadotrophic content of the pituitary gland of tumour-bearing rats employing

bioassay and cytochemical techniques will shortly be in progress. It is hoped that these experiments will provide a better understanding of the role of the pituitary and hormone homeostasis in the widespread constitutional changes seen in tumour-bearing animals.

SUMMARY

1. The effect of nitrofurazone on the testes, seminal vesicle and prostate of normal rats and rats bearing the Walker carcinoma 256 were studied.

2. Nitrofurazone added to the diet of normal adult male rats in a concentration of 0·15 per cent resulted in body weight loss, profound degeneration of the spermatogenic epithelium of the testes and a slight hypertrophy of the seminal vesicle. Hyperplasia of interstitial testicular tissue was not apparent.

3. Atrophic changes in the seminal vesicle consisting of a reduction in height of the secretory epithelial cells, a reduction in the amount of seminal secretion, and a notable increase in fibro-muscular stroma of the gland were associated with growth of the Walker tumour. The degree of atrophy was significantly correlated with the weight of the tumour.

4. Addition of nitrofurazone to the diet of tumour-bearing rats failed to prevent atrophy of the seminal vesicle and prostate glands. Degeneration of the spermatogenic epithelium of the testes was similar to that seen in normal rats.

5. Intraperitoneal administration of 10 mg. of nitrofurazone for 10 days protected tumour-bearing rats against atrophy of the accessory sex organs. This protection was associated with no abnormal change in body weight, nor in weight or histological appearance of the testes.

6. These findings are compared with the previously reported effects of nitrofurazone in the mouse. It is suggested that cytological studies of the pituitary gland of tumour-bearing rats would provide a clearer understanding of the hormonal imbalances associated with the growth of tumours.

The author wishes to record his admiration and respect for the late Professor E. S. Horning under whose direction this study was initiated. I am indebted to Professor A. Haddow, Director of the Chester Beatty Research Institute, for his kindness and consideration in providing facilities with which to carry out this work. The excellent histological preparations are the work of Mr. R. J. McColloch, the photographs that of Mr. K. Moreman and the photographic department of the Chester Beatty Research Institute.

This investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research : Royal Cancer Hospital) from the Medical Research Council, the British Empire Cancer Campaign, the Jane Coffin Charles Memorial Fund for Medical Research, the Anna Fuller Fund, and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

REFERENCES

Begg, R. W.—(1955) Proceedings of the First Canadian Cancer Research Conference, Vol. 1, p. 237. New York (Academic Press Inc.).—(1958) *Advanc. Cancer Res.* **5**, 1.

Idem and Stewart, A. G.—(1952) *Cancer Res.*, **12**, 248.

Haddow, A., Horning, E. S. and Carlton Smith, N.—(1957) *Schweiz. med. Wschr.*, **87**, 396.

Mulinos, M. G. and Pomerantz, L.—(1940) *J. Nutr.*, **19**, 493.
Nelson, W. O. and Steinberger, E.—(1952) *Anat. Rec.*, **112**, 367.
Nissim, J. A.—(1957) *Lancet*, i, 304.
Pearse, A. G. E. and Rinaldini, L. M.—(1950)*Brit. J. exp. Path.*, **31**, 540.
Rinaldini, L. M.—(1949) *J. Endocrin.*, **6**, 54.

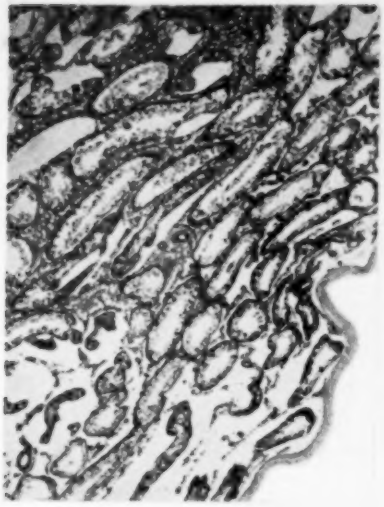
EXPLANATION OF PLATE

Fig. 1*a*.—Testes of a rat fed nitrofurazone 0·15 per cent in the diet for 25 days. Note degeneration of the spermatogenic epithelium and the mucoid substance filling many of the spaces between distorted tubules. Hyperplasia of interstitial tissue is not apparent. ×23.

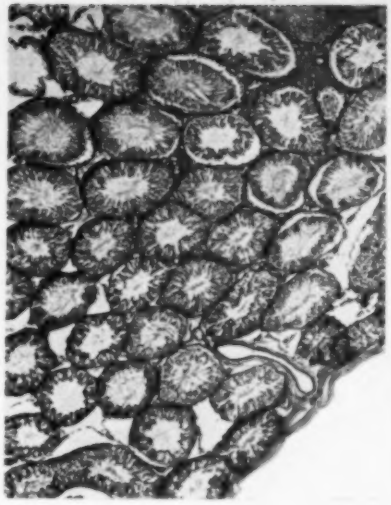
Fig. 1*b*.—Testes of a normal rate. ×23.

Fig. 3*a*.—Atrophic seminal vesicle of a tumour-bearing rat. Note decrease in seminal fluid and increase in fibro-muscular stroma. ×7.

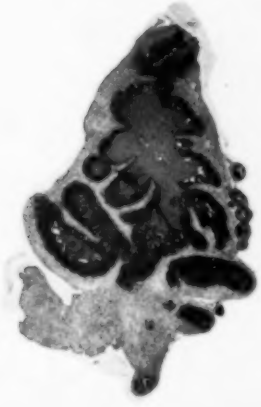
Fig. 3*b*.—Seminal vesicle of tumour-bearing rat treated daily for 10 days with 10 mg. of nitrofurazone intraperitoneally. The relative amounts of seminal secretion and fibro-muscular stroma resemble that of a normal gland. ×7.



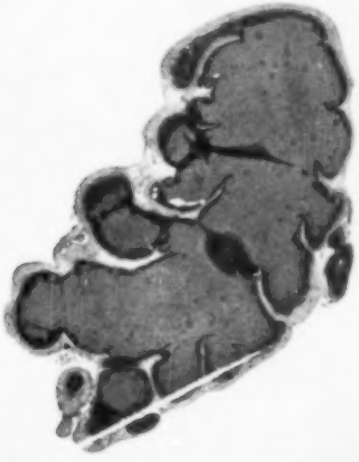
1a



1b



3a



3b

THE INFLUENCE OF DIET ON THE ACTION OF THE WALKER 256 CARCINOMA ON LIVER PROTEIN AND NUCLEIC ACIDS

CATHERINE M. CLARK AND G. A. J. GOODLAD

From the Department of Physiology and Biochemistry, St. Salvator's College, University of St. Andrews, Fife

Received for publication March 19, 1960

In experimental tumour-bearing rats there is evidence that the growing tumour interferes with the metabolism of protein in the tissues of the host (Stewart and Begg, 1953; Allison, Bernstein and Babson, 1954) and that there is a stage in tumour growth when the liver of the host shows a gain in protein content while the carcass as a whole is losing protein (Stewart and Begg, 1953). This effect of a tumour is similar to that obtained by treating rats with cortisone when an increase in both liver protein and nitrogen excretion is observed (Silber and Porter, 1953; Trémolières, Derache and Lowy, 1955; Goodlad and Munro, 1959). The question arises as to whether some aspects of the action of tumours on the metabolism of the host might be due to an increased activity of the adrenal cortex. It has indeed been found that the adrenals of tumour-bearing animals are enlarged (Stewart and Begg, 1953) and in a recent review Begg (1958) has presented evidence to suggest that they may be hyperactive.

In a preceding series of experiments (Goodlad and Munro, 1959) diet was found to modify the action of cortisone on nitrogen metabolism. The increase in protein and ribonucleic acid (RNA) content of the liver induced by cortisone was uninfluenced by the level of protein in the diet but was dependent to a considerable degree on the calorific value of the diet. Liver deoxyribonucleic acid (DNA) content was not influenced by the administration of cortisone under any of these dietary conditions.

In the present work an attempt has been made to carry out a similar study on the effect of diet on the action of the Walker 256 carcinoma on liver composition. It was found that the levels of protein, RNA and DNA were increased in the livers of tumour-bearing animals and that these increases were independent of both dietary protein and calorie intake. A comparison of the distribution of protein and RNA in the various subcellular fractions of the livers of normal and tumour-bearing rats indicated that, although the action of the tumour did not appear to be directed at any one fraction in the case of RNA, there was a significant alteration in the pattern of protein distribution in the tumour-bearing animal, producing a decrease in the proportion of protein found in the mitochondrial fraction. The increase in the DNA content of livers of tumour-bearing rats was not associated with any alteration in the mean DNA content per nucleus of the liver cell, suggesting that the tumour causes an increase in the number of nuclei in the liver.

EXPERIMENTAL METHODS

Animals.—Virgin female albino rats were fasted overnight and those weighing 160–200 g. were selected and housed in individual metabolism cages.

Tumour inoculation.—A specimen of Walker 256 carcinoma, grown intramuscularly in the thigh of an albino rat, was dissected out and a suspension prepared as described by Talalay, Takano and Huggins (1952). 1 ml. portions of this were injected intramuscularly into the right thigh of each experimental animal. Green, Benditt and Humphreys (1950) have demonstrated that dietary protein level influences the growth of the Walker tumour during the induction period but not subsequently and for this reason the rats were fed a diet of adequate protein content for a preliminary period of 6 days. At the end of this period the tumours were easily palpable and rats having tumours of similar size were selected to continue the experiment. A control series of rats from the same stock were fed in parallel.

Diet.—The first series of experiments was designed to study the effect of dietary protein and calorie intake on the response of the liver to the presence of a growing tumour in the host. During the first 6 days (preliminary induction period) the rats were fed a diet adequate in calories and protein content. This was fed in two parts. At 9 a.m., the rats received 1 g. of a vitamin-mineral roughage mixture (Munro, 1949) most of the calories being given in the form of 2·4 g. of starch (potato) and 0·5 g. of glucose. At 5 p.m. a meal providing all the protein and the remainder of the calories in the diet was fed, namely 2·4 g. of casein (unextracted grade, Glaxo Laboratories Ltd., Greenford, Middlesex.), 0·69 g. of glucose and 0·42 g. of fat (margarine). For the remaining 5 days of the experiment the calorific value of the diet was varied by feeding in the morning 1 g. of the vitamin-mineral-roughage mixture either alone (low energy group) or together with 4 g. of starch and 1 g. of glucose (high energy group). The protein content of the diet was varied by feeding in the evening *either* a meal containing 2·4 g. of casein, 0·69 g. of glucose and 0·42 g. of fat (protein-containing group) *or* an isocaloric meal consisting of 1·89 g. of starch, 1·89 g. of glucose and 0·42 g. of fat (protein-free group). In this way, the animals were divided into 8 groups, comprising tumour-bearing or control animals receiving either a protein-containing or a protein-free diet at a high or low calorie intake. On the sixth day of this regimen, the rats were killed by stunning and exsanguination.

In the experiments designed to study the distribution of RNA and protein in the various subcellular fractions of liver and the mean DNA content of liver nuclei, the rats received the diet described above for the preliminary induction period for the duration of the experiment (9–11 days from tumour inoculation), that is all animals received an adequate intake of protein and calories.

Analysis of liver.—The liver, spleen, adrenal glands and tumour were rapidly removed and weighed.

Samples of the liver were analysed for protein N by the Kjeldahl technique. Ribonucleic acid phosphorus (RNAP) and deoxyribonucleic acid phosphorus (DNAP) were determined by the differential solubility of alkaline digests in trichloroacetic acid by methods previously described (Munro and Naismith, 1953). Portions of the liver were taken for histological examination.

Intracellular distribution of RNAP and protein N.—Subcellular fractions were prepared by differential centrifugation in 0·25 M sucrose and their RNAP content

determined as described by Schneider (1948). The protein N content of the different fractions was determined.

DNA content of liver cell nuclei.—Nuclei were prepared by the citric acid procedure, as modified by Smellie, Humphrey, Kay and Davidson (1955). The nuclei, suspended in 0·01 M citric acid, were counted in a haemacytometer; a portion of the suspension was also taken for DNAP determination (Munro and Naismith, 1953) and deoxyribose estimation by the diphenylamine reaction (Schneider, 1957). The DNAP content per nucleus was then expressed in picograms (pg.).

RESULTS

The effect of various dietary conditions on liver, spleen and adrenal weights and tumour growth in rats bearing a Walker 256 carcinoma

The method of tumour implantation described by Talalay *et al.* (1952) for the standardisation of tumour growth was employed in the present work. Although tumour size varied from experiment to experiment, it was relatively constant within any one experiment. Tumour growth appeared to be independent of the various dietary treatments employed (Table I), a finding which is consistent

TABLE I.—*Liver, Spleen and Adrenal Weights of Control and Tumour-bearing Rats under Various Dietary Conditions*

Results are the means of 5 experiments ± S.E. Statistical analysis shows that the spleen and adrenal weights were significantly affected by dietary protein ($P < 0.05$ and $P < 0.01$ respectively); that liver, spleen and adrenal weights were significantly increased in the tumour-bearing animals ($P < 0.01$ in all cases); that the increases in the spleen and adrenal weights caused by the tumour were significantly greater in the rats fed a protein-containing diet ($P < 0.05$ for interactions between tumour and dietary protein intake); that the magnitude of the increase in liver weight was not significantly affected by diet ($P < 0.05$ for interaction of tumour with dietary protein and calorie intakes); that tumour weight was not significantly affected by dietary protein or calorie intake ($P > 0.05$ in both cases for interaction).

Dietary protein	Dietary calorie value	Tumour weight (g.)	Liver weight (g./100 g. of initial body weight)		Adrenal weight (mg./100 g. of initial body weight)		Spleen weight (mg./100 g. of initial body weight)	
			Control series	Tumour-bearing series	Control series	Tumour-bearing series	Control series	Tumour-bearing series
None	Low	11·4±2·8	2·78±0·30	3·14±0·34	31·4±1·4	41·0±4·9	348±62	426±84
	High	9·3±2·2	2·81±0·15	3·61±0·13	29·4±4·3	39·8±4·6	353±41	553±102
Adequate	Low	10·5±2·0	2·64±0·14	3·91±0·35	31·0±3·6	48·2±6·1	393±70	821±142
	High	9·3±2·4	3·20±0·15	4·22±0·68	32·0±2·0	49·4±8·7	374±12	813±133

with the observation of Green *et al.* (1950) who found that growth of the Walker 256 carcinoma was sensitive to the nutritional state of the host only during the first 5–6 days after implantation.

The liver, spleen and adrenals of tumour-bearing rats are larger than those of normal rats (Stewart and Begg, 1953). In the present work increases in the

weights of these organs were observed under all dietary conditions (Table I). The gain in liver weight caused by the tumour was not significantly influenced by diet. The increases in spleen and adrenal weights in tumour-bearing animals did appear to be more marked in the animals receiving a protein-containing diet ($P < 0.05$).

The effect of the Walker 256 carcinoma on the protein and nucleic acid content of rat liver

Stewart and Begg (1953) found that the Walker 256 carcinoma produced liver protein deposition in rats fed a high protein diet. In the present study an increase in liver protein content was observed in tumour-bearing rats, irrespective of the level of protein and energy fed (Table II). This increase in liver protein was

TABLE II.—*A Comparison of the Protein N, RNAP and DNAP Content of the Livers of Tumour-bearing and Control Rats under Various Dietary Conditions*

Results are the means of 6 experiments ± S.E. Analysis of variance shows that the tumour caused significant increases in liver protein, RNA and DNA content ($P < 0.01$ in all cases) and that these effects are independent of dietary protein and calorie intake ($P > 0.05$ for all interactions).

Dietary protein	Dietary calorie value	Liver protein N (mg./100 g. of initial body weight)			Liver RNAP (mg./100 g. of initial body weight)			Liver DNAP (mg./100 g. of initial body weight)		
		Control series	Tumour-bearing series	Mean percentage difference	Control series	Tumour-bearing series	Mean percentage difference	Control series	Tumour-bearing series	Mean percentage difference
None	Low	57·3±5·6	65·1±5·4	+14	2·71±0·19	3·64±0·14	+34	1·07±0·11	1·18±0·08	+10
	High	53·6±6·6	68·5±5·7	+28	2·63±0·14	3·90±0·34	+52	1·04±0·09	1·19±0·02	+16
Adequate	Low	57·4±6·2	85·9±9·4	+50	2·68±0·23	4·37±0·51	+63	1·08±0·10	1·29±0·07	+19
	High	72·0±8·1	86·9±17·9	+21	3·07±0·22	4·49±0·89	+46	1·09±0·10	1·29±0·11	+18

also accompanied by increases in both liver RNA and DNA, protein and calories intake again having no significant effect on the magnitude of these changes (Table II).

In each dietary group the magnitude of the increase in liver RNA content (34–63 per cent) was much greater than that in either liver protein (14–50 per cent) or DNA (10–19 per cent).

Comparison of the intracellular distribution of RNA and protein in the livers of tumour-bearing and control rats

Since the Walker 256 carcinoma produced an increase in both liver RNA and protein content a study of the intracellular distribution of RNA and protein in the livers of tumour-bearing and control rats was undertaken in an attempt to ascertain whether these increases were due to changes in any particular fractions of the liver cell. In the preceding series of experiments it was found that the increases in RNA and protein were independent of the dietary state of the animal.

Consequently in the present study only tumour-bearing and control rats receiving an adequate protein and calorie intake were employed. Table III indicates that, although there was a considerable increase in the amount of RNA in the livers of tumour-bearing aninals ($P < 0{\cdot}1$), the percentage distribution of RNA in the different subcellular fractions was not significantly different from that found in the normal rat liver.

TABLE III.—*The Intracellular Distribution of RNAP and Protein N in the Livers of Control and Tumour-bearing Rats*

The results are the means of 4 experiments ± S.E. in the case of RNAP and 3 experiments ± S.E. in the case of protein N. Statistical analysis ("t" test) shows that the tumour caused a significant increase in total liver RNA content ($P < 0{\cdot}01$); that there was no significant alteration in the pattern of RNA distribution in the subcellular fractions of the livers of tumour-bearing rats; that there was a significant fall in the proportion of protein N present in the mitochondrial fraction of the livers of tumour-bearing animals ($P < 0{\cdot}05$).

Treatment	Total amount per liver (mg./100 g. of initial body weight)	Distribution in the subcellular fractions (expressed as a percentage of the whole homogenate)				
		Nuclear	Mitochondrial	Microsomal	Soluble	Recovery
RNAP—						
Control	3·01±0·03	7·6±0·9	13·5±1·4	49·6±2·0	25·1±1·9	95·8±2·3
Tumour-bearing	5·64±0·61	9·1±0·9	11·5±3·3	45·7±2·8	28·7±5·5	95·0±2·8
Protein N—						
Control	74·5±0·7	15·9±0·3	28·6±2·8	18·6±3·0	35·6±3·4	98·6±6·5
Tumour-bearing	88·3±6·7	20·5±3·8	13·7±3·6	19·9±3·3	39·9±4·4	94·0±1·8

The protein data (Table III) suggest that there was again an increase in the protein content of the livers of tumour-bearing animals. However due to the variation between animals this increase did not attain statistical significance. Nevertheless the pattern of distribution of protein in the different subcellular fractions was significantly altered by the presence of a tumour, namely by a fall in the proportion of the cellular protein present in the mitochondrial fraction ($P < 0{\cdot}05$).

Mean DNA content of nuclei isolated from the livers of control and tumour-bearing rats

Since the total DNA content of the livers of tumour-bearing rats was increased, a study was made to determine whether this rise was accompanied by an increase in the mean nuclear DNAP. The dietary conditions were similar to those described in the preceding section. DNAP was estimated both by phosphorus determination following the Schmidt and Thannhauser procedure (1945) for the isolation of DNA and by the diphenylamine method described by Schneider (1957).

Table IV shows that although the total DNA content of the liver is considerably increased in tumour-bearing animals ($P < 0{\cdot}01$) the mean DNA content per liver nucleus did not differ from that obtained in normal rats.

TABLE IV.—*A Comparison of the Mean DNAP Content of Liver Cell Nuclei in Control and Tumour-bearing Rats*

Results are the means of 7 experiments ± S.E. Statistical analysis ("t" test) shows that there was a significant increase in the DNAP content of livers of tumour-bearing rats ($P < 0.01$) but no alteration in the mean DNAP content of the nucleus.

Treatment	Tumour weight (g.)	Liver DNAP (mg./100 g. of initial body weight)	DNAP (pg./nucleus) as determined by	
			Phosphorus estimation	Deoxypentose estimation
None	—	0·97±0·02	0·96±0·02	0·90±0·03
Tumour	9·9±0·8	1·20±0·06	0·95±0·04	0·90±0·03

Histological examination of the livers of control and tumour-bearing rats

Histological examination of the livers of tumour-bearing rats failed to show any evidence of centres of haematopoiesis such as have been described by Ventura, Richer and Selye (1957) in livers of rats bearing large Walker tumours (ca. 40 g.).

DISCUSSION

In the present series of experiments it has been found that the Walker 256 carcinoma causes an increase in the weights of the liver, spleen and adrenal glands of the host. The increase in liver size was independent of the level of protein and calorific value of the diet while the increases in spleen and adrenal weights were more marked in rats receiving a protein-containing diet. Tumour growth also appeared to be independent of the dietary conditions employed.

Examination of liver composition showed that there was an increase in the total amounts of protein, RNA and DNA in the livers of tumour-bearing rats, irrespective of the protein and calorie content of the diet. The fact that even the livers of tumour-bearing rats receiving a protein-free diet showed this increase suggests that the protein laid down in the liver arises from the breakdown of carcass protein which is known to occur in tumour-bearing animals (Sherman, Morton and Mider, 1950; Begg and Dickinson, 1951). It is of interest to note that Green *et al.* (1950) could not demonstrate any increase in the protein content of the livers of rats bearing the Walker 256 carcinoma when these animals had been extensively depleted of body protein by feeding them a protein-free diet for three weeks before tumour inoculation.

This action of the tumour in causing an increase in liver protein by depleting the carcass appears similar to the action of cortisone. There is evidence that this hormone acts primarily on the carcass causing a breakdown of tissue protein resulting in an increased supply of amino acids to the liver (Goodlad and Munro, 1959). Due to the sensitivity of the growth of the Walker 256 carcinoma to dietary protein (Green *et al.*, 1950) the dietary conditions employed in the present work were not exactly similar to those of a preceding study of the action of diet on the response of the liver to cortisone adminstration (Goodlad and Munro, 1959). Nevertheless there would appear to be certain differences in the response of the liver to these two circumstances which are not related to the change in experimental conditions. In the first place, it was found that the deposition of liver protein and RNA caused by cortisone was related to the calorie intake, whereas the increase of these two constituents in the livers of tumour-bearing rats did not

vary with calorie intake. Secondly, although cortisone was without effect on liver DNA, the tumour caused a significant rise in the total content of DNA in the liver. Finally, the relative increase in liver RNA with respect to the increase in liver protein was much greater in the tumour-bearing rats than in the cortisone-treated rats. These results therefore suggest that the changes observed in liver composition in tumour-bearing animals may be brought about by a different mechanism from that by which cortisone exerts its effect.

Studies on the intracellular distribution of RNA and protein showed that, while the RNA of all subcellular fractions was increased by the action of the tumour, the percentage of the total liver protein present in the mitochondrial fraction was lowered in tumour-bearing animals. Whether this was due to a decrease in the amount of protein per mitochondrion or to a decrease in the number of mitchondria relative to the other cellular components cannot be decided from the data available. Allard, de Lamirande and Cantero (1953) have in fact found that the mitochondrial population (expressed as mitochondria per cell) of liver cells adjacent to a growing hepatoma but themselves free of tumour was significantly lower than that of normal liver cells.

The total DNA content of the livers of tumour-bearing rats was significantly increased. A rise in liver DNA may be attributed to one of the following :—

(*a*) Centres of extramedullary haematopoiesis may be present. These have been observed in the liver and other organs of tumour-bearing animals (Begg, 1958).

(*b*) The degree of polyploidy of liver nuclei may have increased. Leuchtenberger, Leuchtenberger and Myeki (1958), working with mice, showed that the amount of DNA per liver nucleus increased folloing injection of tumour DNA.

(*c*) There may be formation of new liver nuclei.

Thomson, Heagy, Hutchison and Davidson (1953) have investigated the mean DNA content per nucleus of cells isolated from different tissues of the rat. They found that, while the mean DNAP content of liver nuclei was of the order of 0·90 pg. per nucleus or greater, that of spleen and bone marrow tissues, where active haematopoiesis occurs, was only 0·63 pg. and 0·67 pg. per nucleus respectively. Leucocyte nuclei had a mean DNAP content of 0·64 pg. per nucleus. Thus a fall in the mean DNA content of liver cell nuclei might be expected if infiltration of haematopoietic centres was occurring to any great extent. An increase in polyploidy, on the other hand, would be reflected by an increase in the mean nuclear DNA content. The fact that the DNA content of liver cell nuclei was not altered and the absence of any histological evidence of haematopoietic centres suggest that the rise in total liver DNA in tumour-bearing rats is due mainly to the formation of new nuclei. This view receives further support from the report that resting liver mitosis rates in rats bearing the Walker 256 carcinoma were seven times greater than normal (Hemingway, 1960).

SUMMARY

1. The effect of the Walker 256 carcinoma on the tissues of the host has been studied in rats receiving diets varying in protein and calorie content.

2. Tumour growth was found to be independent of the various dietary treatments employed.

3. The liver, spleen and adrenals of tumour-bearing rats were larger than those of normal rats. This increase in weight was independent of the level of protein

and calorie content of the diet in the case of liver but the rises in spleen and adrenal weights observed in tumour-bearing animals were more marked in rats receiving a protein-containing diet.

4. An increase in the protein, RNA and DNA content in the liver was observed in tumour-bearing rats, under all dietary conditions. The percentage increase in RNA being much greater than that of either protein or DNA.

5. The intracellular distribution of RNA in liver was unaffected by the presence of a tumour. However protein distribution was altered in the livers of tumour-bearing rats, there being a fall in the proportion of protein found in the mitochondrial fraction.

6. The mean DNA content of liver nuclei in rats bearing the Walker carcinoma did not differ from that observed in normal rat liver.

The authors wish to express their gratitude to Dr. H. N. Munro of the Department of Biochemistry, Glasgow University, for valuable advice and discussion, Professor A. Haddow F.R.S. of the Chester Beatty Research Institute, London, for supplying a suitable strain of rats and samples of the Walker 256 carcinoma and Dr. W. W. Park of the Department of Pathology, St. Andrew's University, for advice on certain histological points. One of the authors (G. A. J. G.) carried out part of this work during the tenure of a Beit Memorial Fellowship at the Department of Biochemistry, Glasgow University, and would like to thank Professor J. N. Davidson, F.R.S., for facilities granted. The latter stages of this work were supported by a grant from the Medical Research Council for expenses and scientific assistance to one of us (G. A. J. G.) for which we are most grateful.

REFERENCES

ALLARD, C., DE LAMIRANDE, G. AND CANTERO, A.—(1953) *Canad. J. med. Sci.*, **31**, 103.
ALLISON, J. B., BERNSTEIN, E. H. AND BABSON, A. L.—(1954) *Fed. Proc.*, **13**, 174.
BEGG, R. W.—(1958) *Advanc. Cancer Res.*, **5**, 1.
Idem, AND DICKINSON, T. E.—(1951) *Cancer Res.*, **11**, 409.
GOODLAD, G. A. J. AND MUNRO, H. N.—(1959) *Biochem. J.*, **73**, 343.
GREEN, J. W., BENDITT, E. P. AND HUMPHREYS, E. M.—(1950) *Cancer Res.*, **10**, **769**.
HEMINGWAY, J. T.—(1960) *Nature, Lond.*, **185**, 106.
LEUCHTENBERGER, C., LEUCHTENBERGER, R. AND MYEKI, E.—(1958) *Proc. nat. Acad. Sci., Wash.* **44**, 700.
MUNRO, H. N.—(1949) *J. Nutr.*, **39**, 375.
Idem AND NAISMITH, D. J.—(1953) *Biochem. J.*, **54**, 191.
SCHMIDT, G. AND THANNHAUSER, S. J.—(1945) *J. biol. Chem.*, **161**, 83.
SCHNEIDER, W. C.—(1948) *Ibid.*, **176**, 259.
Idem—(1957) in " Methods in Enzymology ", vol. 3, p. 680, Ed. by Colowick, S. P. and Kaplan, N. O., New York (Acad. Press Inc.).
SHERMAN, C. D., MORTON, J. J. AND MIDER, G. B.—(1950) *Cancer Res.*, **10**, 374.
SILBER, R. H. and PORTER, C. C.—(1953) *Endocrinology*, **52**, 518.
SMELLIE, R. M. S., HUMPHREY, G. F., KAY, E. R. M. AND DAVIDSON, J. N.—(1955) *Biochem. J.*, **60**, 177.
STEWART, A. G. AND BEGG, R. W.—(1953) *Cancer Res.*, **13**, 556, 560.
TALALAY, P., TAKANO, G. M. V. AND HUGGINS, C.—(1952) *Ibid.*, **12**, 834.
THOMSON, R. Y., HEAGY, F. C., HUTCHISON, W. C. AND DAVIDSON, J. N.—(1953) *Biochem. J.*, **53**, 460.
TRÉMOLIÈRES, J., DERACHE, R. AND LOWY, R.—(1955) *Ann. Nutr., Paris*, **9**, 179.
VENTURA, J., RICHER, C-L. AND SELYE, H.—(1957) *Cancer Res.* **17**, 215.

THE *IN VITRO* CYTOTOXIC ACTIVITY OF MURINE ISO-ANTISERA ON NORMAL AND MALIGNANT CELLS*

PATRICK O'GORMAN†

From the Department of Experimental Pathology, Guy's Hospital Medical School

Received for publication April 13, 1960

In mice passive immunity to leukaemic homografts has been obtained by transfer of sera containing iso-antibodies against both the H–2 antigens and the so-called X-type antigens, examples of which have been found in several leukoses (Amos and Day, 1957 ; Gorer and Amos, 1956 ; Gorer, personal communication). Histologically, leukaemic grafts can be seen to be destroyed by a massive exudation of fluid into the graft on the fourth and fifth day ; thereafter host cells appear which act only as scavengers (Gorer, 1958). Sarcomatous grafts, on the other hand, are invaded by a syncytium of host cells which engulf and destroy the graft. The antigens of the H–2 system are the major antigenic factors controlling the fate of homografts in mice, and are determined by a long series of alleles on the ninth chromosome. Present knowledge of this system has been reviewed by Gorer and Mikulska (1959).

These results suggested that iso-antibodies play an important part in homograft rejection, particularly of leukotic grafts, and might have a detectable effect *in vitro*. There have been few purely *in vitro* studies of this effect. Miller (1956) failed to show any effect upon cells growing in tissue culture of exposure to iso-antibody ; the cells were of epithelial origin and the presence of active complement was not ensured. Mouse serum is usually devoid of complementary activity *in vitro*, and Ritz (1911) stated that mouse serum was deficient in the " end-piece " of complement, whilst Brown (1943) believed it to lack the second component, C'2. McGhee (1952) demonstrated murine complement *in vitro* and convincing evidence of its activity *in vivo* has been provided by the experiments with diffusion chambers reported by Algire, Weaver and Prehn (1957), who obtained lysis of Hela cells and by Amos and Wakefield (1958) who demonstrated lysis of mouse leukotic cells.

Schrek (1936) has described a method of distinguishing viable and non-viable cells in a suspension by means of differential staining, using eosin, methylene blue and trypan blue, dyes which stain non-viable cells, whilst viable cells are not affected, as Pappenheimer (1917) had shown many years before. A technique for studying the effect of murine iso-antisera *in vitro* was briefly reported by Gorer and the present author (Gorer and O'Gorman, 1956) which used Schrek's method. The technique did not prove completely reliable in further use and a more satisfactory modification is now presented, together with a detailed study of the nature of the antibody, of the antigenic system involved and of the effect of

* This work has formed part of a thesis accepted by the University of London for the degree of Doctor of Medicine.

† Present address : Group Laboratory, Lewisham Hospital, London, S.E.13.

cytotoxic iso-antibody upon normal leucocytes and upon several leukoses and two sarcomata.

MATERIALS

Mice.—The mice used were from the inbred lines Strain A, BALB/c, C_3H and C57Bl, maintained in the laboratory of Dr. P. A. Gorer, and also the progeny of the backcross (A × C57Bl) F, × C57Bl.

Complement.—Guinea-pig complement was used, obtained by cardiac puncture and stored at −20° C. until needed.

Tumours.—Two ascites leukoses were used, EL4 (C57Bl strain) and CL2 (BALB/c). EL4 has been maintained by transplantation for many years, and will not grow subcutaneously in foreign strains, but sometimes will do so intraperitoneally. CL2 is of recent origin and is strain specific. Two other leukoses CL1 (BALB/c) and EL8 (C57Bl) are of recent origin and are strain specific, but produce generalized leukoses with massive enlargement of the spleen. Three ascites sarcomata were used, BP8 (C_3H strain) and Sa1 and MTC 1A (both A strain). All of these tumours sometimes grow progressively if inoculated intraperitoneally in large doses into foreign strains. In moderate dosage, subcutaneously, they are strain specific. Three other strain specific tumours were used—the mammary carcinomata EMT (C57Bl), AMT (A strain) and CT1 (BALB/c) and the leukosis EL5; all were maintained as solid subcutaneous growths.

Iso-antisera.—Details of the hyper-immune iso-antisera used will be given at the appropriate places in the text.

Dyes.—Both eosin and trypan blue were used at a concentration of 1/2000 in Tyrode's solution, filtered after preparation. No significant difference was observed in the results of parallel experiments using the two dyes, but trypan blue gives more clear-cut staining.

METHODS

Preparation of cell suspensions

1. *From ascites tumours.*—The ascites was diluted in Ringer's solution in the case of the non-haemorrhagic leukotic ascites, and in 3 per cent sodium citrate in the case of the haemorrhagic sarcomatous ascites. Total cell counts were made at once, and the dilution adjusted with normal mouse serum and Ringer's solution to give a final count of approximately 30,000/mm.^{3} in 1 : 5 normal mouse serum. Viability counts gave from 0–5 per cent stained cells in the case of the ascites leukoses, and about 0–10 per cent in the ascites sarcomata.

2. *From spleen.*—The same method was used for both normal and leukotic spleens. The animal was killed with chloroform, the abdomen opened, the spleen exposed and the splenic artery and vein were cut with fine scissors. A 10 ml. syringe was fitted with a fine intra-dermal (No. 26 G.) needle and filled with sterile Ringer's solution. The point of the needle was introduced under the capsule of the spleen and light pressure applied to the plunger. Immediately a small white area appeared around the needle point, which extended as the pressure was maintained. After about 1 ml. had been injected the needle was withdrawn and reinserted in a different position. In this way the whole spleen was gradually perfused. If the splenic vessels are not divided, the liver and kidneys can be

perfused at the same time, and this is probably a more convenient way of perfusing these organs than by cannulation of the appropriate artery or by intra-cardiac injection. After perfusion the spleen was excised, freed from fat and finely minced with small scissors and suspended in 1 : 5 normal mouse serum. Small clumps were broken up by gentle pipetting, and the larger clumps which remained were allowed to settle out. The concentration was adjusted to a final figure of approximately 30,000 leucocytes/mm.^{3} Viability counts usually showed about 5–10 per cent stained cells.

Titration of complement

Undiluted guinea-pig serum was often toxic to the target cells whilst its complementary activity declined rapidly on dilution. It was therefore titrated in terms of its ability to complement the cytotoxic activity of iso-antisera. A serum known to be toxic for the target cells was used at a dilution of 1 : 5 and the complement was titrated in the dilution range 1 : 1 to 1 : 4 ; it was seldom active at greater dilutions. Controls of normal serum and another iso-antiserum known not to be toxic for the target cells were used.

Antibody titration

The cytotoxic activity of an antiserum was usually investigated in the form of a titration, to exclude zoning.

Doubling dilutions of each antiserum to be tested were made in 1 : 5 normal mouse serum. Control tubes, containing only the diluted normal serum, were included. A volume (0·05 ml.) of cell suspension was added to each tube and finally a volume of diluted complement. The rack was incubated at 37° C. for 45–75 minutes, except in the experiments on the timing of the reaction. After incubation dye was immediately added to all the tubes to make a final dilution of 1 : 10. The tubes were left for a few minutes before counting to ensure correct staining.

Two methods of reading the titration were employed. In the first, differential counts were made on each tube. In the second, the two control tubes were counted and the cells from the rest of the row were streaked across a glass slide and inspected. The results were expressed as +++, ++ etc., the term AC being used when the effect was almost complete and there were virtually no viable cells to be seen ; a negative was scored when the number of stained cells approached that in the control tubes. This method is entirely satisfactory for the routine titration of a serum, but the method expressing the result as a percentage viable count was found to be more delicate when comparative work was being done. Results obtained by the two methods are strictly comparable.

Haemagglutination

The " human serum : dextran " technique of haemagglutination described by Gorer and Milulska (1954) was used for genetic studies and titration of sera.

In vivo *absorption*

Amos (1955) has described the *in vivo* absorption of antibody by mice possessing the appropriate antigen. If a potent serum such as BALB/c anti-C57Bl, is

injected intra-peritoneally into C57Bl mice, the haemagglutinin titre in the recipients' serum falls rapidly and will not be detectable after two hours, even if as much as 0·5 ml. of antiserum is injected. The absorption in all cases is specific and non-specific absorption does not occur.

RESULTS

The morphology of the damaged cells

There was considerable variation in the appearance of the stained cells. Whilst some retained a normal rounded outline, in which the nucleus could barely be seen, other cells became swollen, sometimes grossly so. The outline of these cells became irregular and indistinct and occasionally the cell membrane appeared to have developed bubbles. In the more severely affected cells the nucleus could be clearly seen when trypan blue was used as the stain. Actual lysis of the cells was not seen.

Effect on normal and malignant cells

No significant difference could be detected between the results obtained using normal or leukotic white cells, but ascites leukoses were preferable as the suspensions contained less debris and fewer stained cells than those made from spleen or sarcomatous ascites.

The effect of heat

Heating the antiserum at 56° C. for 30 minutes was without effect on the titre.

Rate of reaction

Murine iso-antisera appear to be toxic to homologous cells even after very short exposure, in the presence of active complement. In an experiment in which BALB/c anti-BP8 was tested on normal C_3H leucocytes, 90 per cent of the target cells were stained after only 10 minutes' incubation at 37° C. The control at this stage contained only 5 per cent non-viable cells. After 20 minutes' incubation 95 per cent of the cells were dead; the increase is probably not significant. On the other hand, prolonged incubation, i.e. for more than $1\frac{1}{2}$ hours produced high numbers of dead cells in the control tubes.

Time of appearance of cytotoxic antibody

Groups of four mice were bled on successive days after inoculation with tumour suspension. The blood from the individuals of each group was pooled and the cytotoxic activity of the serum tested on appropriate cells. The haemagglutinating titre was determined at the same time.

The results of one such experiment are expressed graphically in Fig. 1. The animals were BALB/c, inoculated subcutaneously with EL4. The sera were tested for cytotoxicity on EL4, and for haemagglutinating activity on A and C57Bl red cells. As can be seen from the graphs neither cytotoxin nor haemagglutinin was present on the eighth day after inoculation. Both appeared on the ninth day, and the titres rose together on the same day.

The Nature of the Antigenic System

Absorption, in vivo, of anti-X

In the initial stages of this investigation it was thought that the antigen involved was the so-called antigen X described by Gorer and Amos (1956), to which reference has already been made. This hypothesis was tested by absorption experiments, using the method of *in vivo* absorption described by Amos (1955). One such

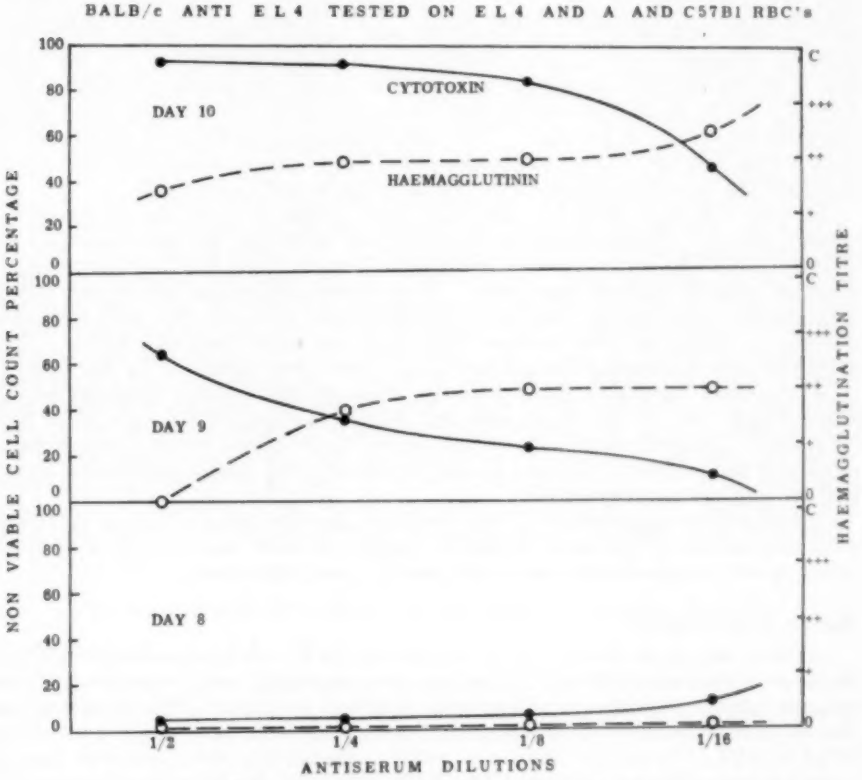


FIG. 1.—Time of appearance of iso-antibody.

experiment is illustrated in Table I. An injection of 1·0 ml. of a sample of BALB/c anti-EL4 was given intra-peritoneally to each of three BALB/c mice and three (C57Bl × A) F_1 mice. These animals were bled out 24 hours later and the sera from each group were pooled and tested on EL4, in parallel with normal mouse serum. BALB/c anti-EL4 serum contains the H–2 antibodies anti-E and anti-D^b, and these should not be absorbed by passage in BALB/c. They should be absorbed, however, by passage in C57Bl, or C57Bl F_1, and this in fact did occur. When tested by haemagglutination the F_1 passaged serum gave a

TABLE I.—*Absorption* in vivo *of Cytotoxic Antibody*

BALB/c anti-EL4 passaged in	Percentage non-viable cells at antiserum dilutions of: 1/1	1/2	1/4	1/8	Haemagglutinin titres: A strain	C57Bl
BALB/c	71	93	100	100	1 : 4096	1 : 64
(C57Bl × A)F_1	1	4	8	5	1 : 256	0

titre of only 1 : 256 for strain A cells (which would detect anti-E) and no titre for C_3H cells (also anti-E) nor for C57Bl cells (anti-D^b and anti-E) whilst the BALB/c passaged serum gave titres of 1 : 4096 for A cells, 1 : 512 for C_3H and 1 : 64 for C57Bl. As can be seen from the table, the serum passaged in the (C57Bl × A) F_1 showed no residual cytotoxic activity for E14 tumour cells killing a maximum of only 8 per cent. The serum passaged in BALB/c on the other hand killed 100 per cent of the target cells. The cytotoxic antibody had been absorbed out in a similar manner to the haemagglutinating antibody.

Sera of this type (BALB/c anti-EL4) when tested *in vivo*, can be shown to contain anti-X. This antibody is not absorbed when the serum is passaged in C57Bl or C57Bl F_1, and should therefore have been detected in this experiment, if it was in fact the antibody concerned. Even a short absorption left no detectable cytotoxicity. 0·2 ml. of BALB/c anti-EL4 was injected into BALB/c and (C57Bl × A) F_1, and the animals were bled out after only two hours. The cytotoxic and haemagglutinating antibodies were both absorbed by the (C57Bl × A) F_1, there being no residual activity, although the BALB/c passaged serum killed 80 per cent of the target cells and gave a haemagglutinin titre of 1 : 256 for A cells. The difference between the titre of the absorbed serum in this experiment and that shown in Table I is due to the different amounts of serum given (0·2 ml. and 1·0 ml. respectively).

These results suggested that both cytotoxic and haemagglutinating antibody were determined by the same antigenic system, in other words the H–2 system, and a series of experiments was undertaken to prove this theory.

Backcross experiments

A cross was made between A × C57Bl and the F_1 was backcrossed to C57Bl. In these crosses some of the H–2 genes are segregating, and 50 per cent of the progeny of the backcross will have the A strain genotype. The A strain has the H–2 constitution CDEFHKRWYZ whilst the C57Bl can be written as D^bEFK^bRVZ (Gorer and Mikulska, 1959). Restricting the discussion to the pertinent genes, the (A × C57Bl) F_1 is CDK/D^bK^b and the B backcross are either D^bK^b/D^bK^b or CDK/D^bK^b. Tissues of the animals carrying CDK will naturally absorb the appropriate antibodies from sera.

The progeny of the B backcross were tested for the presence of these antigens by haemagglutination and divided into H–2 positive (i.e. carrying CDK) and H–2 negative (lacking CDK). Equal numbers of H–2 positive and negative animals were tested by the technique of *in vivo* absorption (Amos, 1955). All were given an intra-peritoneal injection of 0·1 or 0·2 ml of C57Bl anti-MTC serum; MTC was an A strain sarcoma and this serum contained anti-C, anti-D and anti-K.

The mice were bled after 18 hours and the sera tested for cytotoxicity. An excellent fit was obtained with the results of typing by haemagglutination. Of

12 B backcross mice 6 were H–2 positive and 6 were H–2 negative; none of the sera of the H–2 positive group showed any residual cytotoxic activity, whilst those of all the H–2 negative mice showed marked cytotoxicity after absorption. No discrepancies were found.

The cytotoxic antibodies, anti-D and anti-K^b

Anti-D^k.—Sera of the type A anti-C_3H (H–2a anti-H–2k) can be shown to contain haemagglutinins only erratically but are toxic for C_3H leucocytes and sometimes for the C_3H sarcoma BP8. A sample of H–2a anti–H–2k was kindly prepared by Dr. George Snell in the I–R strains AKK and AKR established by him. These strains differ only at their H–2 sites, and so any antibody formed must be against an H–2 component. This serum was also toxic for C_3H leucocytes, killing 74 per cent, as against 4 per cent in the control. A sample of H–2a anti-H–2k prepared in this laboratory killed 90 per cent of the leukotic cells. Neither serum was toxic for BALB/c or C57Bl leucocytes. Gorer and Mikulska (1959) quote evidence from their crossover combinations to show that this new antigen should be on the D part of the chromosome and propose the tentative label D^k.

Anti-K^b.—The antigenicity of the alleles of K was investigated using the crossover combinations described by Gorer (1956). An antiserum was prepared in the D—K+ (H–2h) crossover against the C57Bl (H–2b) tumour EL4. It was found to be toxic for cells from C57Bl and the D+K— (H–2i) crossover, but was not toxic for the B+E— (H–2g) crossover. The D+K— crossover could only have acquired its antigen in the K position from H–2b, whilst the B+E— crossover could have acquired this antigen from either H–2b or H–2d. If it had come from H–2b then this serum should have been toxic for the B+E— cells. That the reverse happened shows that there must be two different antigens in the K position on H–2b and H–2d, and the newly identified antigen on H–2b has been termed K^b. The effect could not be due to an antigen in the C position, for it could only have come from H–2b and the serum would therefore have been toxic for B+E— cells.

The Susceptibility of Leukoses and Sarcomata to Cytotoxic Antibody

A. *The leukoses*

EL4.—Very many experiments were performed in which EL4 cells were used as the target. This leukosis is highly susceptible to antibody, and typically 90–100 per cent of the EL4 cells are killed against 1–5 per cent in the control tubes.

EL8.—Suspensions of leukotic cells made from a spleen invaded by this leukosis contain few stained cells. It is fully susceptible to the action of antibody. When tested with BALB/c anti-EL4 non-viable cell counts of between 84 and 96 per cent were obtained depending on antibody dilution, whilst the control tubes showed 16–19 per cent stained cells.

CL1 and CL2.—CL1 produces massive splenic enlargement, and CL2 is an ascites leukosis. In an experiment in which CL1 and EL4 were tested in parallel with BALB/c anti-EL4 and A anti-CT1, the following results were obtained. The BALB/c anti-EL4 killed 100 per cent EL4 cells but only 18 per cent CL1; on the other hand, the anti-CT1 killed only 2 per cent EL4 and 69 per cent CL1. Both sera were used at a dilution of 1 : 1. In another experiment the BALB/c

leukoses were tested with three different sera, namely A anti-EL4, C57Bl anti-BALB/c and A anti-CT1. A anti-EL4 showed some slight toxic activity against CL1, for at a dilution of 1 : 2 it killed 20 per cent of the cells, compared with 10 per cent in the control tubes; differences of this order may not be significant. The two anti-BALB/c sera produced 75 and 73 per cent stained cells respectively. In the case of CL2 there was no difference in the non-viable cell counts in the A anti-EL4 and control tubes. This leukosis if anything was slightly more susceptible to the cytotoxic effect of the anti-BALB/c sera, the figures being 81 and 86 per cent in the two cases.

B. *The sarcomata*

Details of the two sarcomata BP8 (C_3H) and Sa1 (A strain) have been given above. There was a striking difference between the results obtained with the two tumours.

BP8.—Most of the experiments on BP8 used samples of BALB/c anti-BP8, but A anti-BP8 was also included; sera of the latter type contain anti-D^k and have been discussed above. The BP8 tumour cells are not as susceptible as leukotic or normal leucocytes to the action of antibody; in most experiments non-viable cell counts were of the order of 40 to 60 per cent at the lower dilutions of antibody and on only one occasion did the count exceed 70 per cent. The difference between the susceptibilities of BP8 and normal C_3H leucocytes is shown in Table II, which illustrates an experiment in which both were tested with A

TABLE II.—*Cytotoxic Effect on BP8 and Normal Leukocytes*

	Percentage non-viable cells at antiserum* dilutions of:							
Target cells	Controls		1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64
BP8 . . .	23	21	55	63	51	52	30	16
CH_3 leucocytes .	14	11	96	92	95	93	81	44

* Antiserum was A anti-BP8—see text.

anti-BP8. When tested with BALB/c anti-BP8 in parallel with EL4, BP8 showed less than 45 per cent stained cells, whereas the leukosis showed 95 per cent.

Sa1.—Sarcoma 1 appears to be completely insensitive to the *in vitro* cytotoxic activity of iso-antisera, and despite repeated testing, with different sera, no convincing evidence was obtained of any toxic effect. A strain leucocytes are as fully sensitive to antibody as are the leucocytes of any other strain. Table III shows the results of tests made in parallel on Sa1 and A strain leucocytes with

TABLE III.—*Cytotoxic Effect on Sarcoma 1*

	Percentage non-viable cells at antiserum dilutions of:						
Anti-serum	Controls		1 : 2	1 : 4	1 : 8	1 : 16	Target cells
C_3H anti-AMT	1	2	5	3	7	2	Sa1
	8	6	90	85	56	9	A strain leucocytes
C57Bl anti-MTC	—	—	7	9	3	8	Sa1
	—	—	95	89	91	90	A strain leucocytes
BALB/c anti-BP8	—	—	1	6	10	11	Sa1
	—	—	91	95	96	91	A strain leucocytes

three different sera, all of which contained antibodies toxic for A strain white cells. AMT and MTC are both A strain tumours, whilst BALB/c anti-BP8 contains anti-E and anti-K both of which are toxic for A strain leucocytes.

Results of an experiment in which one anti-serum was tested on cells of one leukosis and the two sarcomata are given in Table IV, and provide striking

TABLE IV.—*Cytotoxic Effect upon Three Tumours*

Target cells	Percentage non-viable cells at antiserum* dilutions of: Controls		1 : 2	1 : 4	1 : 8	1 : 16
EL4 . .	16	12	95	98	95	91
BP8 . .	1	6	61	49	56	61
Sa1 . .	3	2	5	7	3	9

* Antiserum was BALB/c anti-BP8.

evidence of the difference in susceptibility to antibody of the cells of the three tumours.

DISCUSSION

The function of circulating antibody in homograft rejection has been the subject of much discussion. By technical modifications of Gorer and Mikulska's (1954) method H–2 antibodies have now been detected in mice before the histological onset of the homograft reaction (Gorer, Mikulska and O'Gorman, 1959) and the demonstration in the present work that these antibodies have a cytotoxic effect on leukoses and sarcomata is evidence against a purely cellular mechanism. The cytotoxic technique is not as sensitive in antibody detection as is the human serum : dextran method, but has been the means of identifying two "new" H–2 antigens, K^b and D^k.

No evidence was found of any *in vitro* cytotoxic effect of the anti-X described by Gorer and Amos (1956). This may be because anti-X is not toxic under these experimental conditions, or because anti-X is not a cytotoxic but a cytostatic antibody, holding the graft in check until the body's defences complete the rejection.

Circulating antibody plays a much more decisive role in the destruction of leukotic grafts than in any others, and for this reason it would seem that leukotic cells or normal white cells would be the material of choice for the investigation of the action of antibody. It is unfortunate that Algire and his co-workers did not include such cells among the targets which they placed in their diffusion chambers. Cells such as those of mammary carcinomata may be completely insensitive to the action of antibody, and in any case it is essential to ensure an adequate level of antibody within the chamber, antibody which has not been produced there by host cells. Amos and Wakefield (1958) having ensured an adequate antibody level have been able to demonstrate lysis of leukotic cells within chambers from which host cells had been excluded.

Lysis of target cells has not been observed in the present work, and usually the cell morphology is little affected. This absence of cytolysis *in vitro*, of course, may not be an accurate reflection of *in vivo* conditions, but if it is accurate then it conforms very well with the histological appearances of regressing grafts. Histiocytic invasion and phagocytosis follow the exudation of fluid into leukotic grafts,

and it is only in such grafts that cytolysis is observed before this invasion. In grafts of sarcomata little or no cytolysis occurs before the invasion of the graft by the syncytium of host histiocytes. The function of the circulating antibody may not be the destruction of the graft, but rather the killing of the graft cells to prepare them for the phagocytes which have yet to come, and the differences in the homograft reactions elicited by different tumours (or tissues) may be a reflection of their differing susceptibility to the toxic action of antibody.

Certainly, most marked differences were found in the antibody susceptibilities of the various tumours which were used in the present work. The sarcomata do not show the uniform susceptibility of the leukoses, and Sa1 appears to be completely insensitive to the toxic action of antibody *in vitro*, although it does have an effect *in vivo*. BP8 is susceptible to the toxic action of antibody *in vivo* as has been shown by the neutralization technique (Gorer, 1956) and by passive immunity studies, but only partly so *in vitro*.

Snell (1957) has propounded a classification of transplantable tumours into three types, based on their susceptibility to the action of antibody. The first class comprises tumours which are highly susceptible and includes Gorer's A strain leukaemia, the BAGG rat lymphosarcoma, the Brown-Pearce carcinoma (Kidd's subline) EL4 and other tumours, all of lymphoid origin. Not only EL4 but also the other leukoses used in the present work have been shown by Gorer to be susceptible to the action of antibody by passive immunity studies, and they are all susceptible to the cytotoxic effect *in vitro*. Snell's second category comprises tumours with low but demonstrable susceptibility to humoral antibodies, and includes Sa1 and the lymphosarcoma $6C_3HED$. These tumours respond to the action of antibody by enhanced rather than inhibited growth when passive transfer of antiserum is used ; $6C_3HED$ has been found to be slightly and irregularly susceptible to the toxic effect of antibody by the neutralization technique (Mitchison, 1955). The *in vitro* results confirm Snell's (1957) separation of EL4 and Sa1 into different categories, but it is difficult to allocate BP8 to one or the other, for pre-treatment of the host with antiserum produces passive immunity or enhancement of the tumour depending on the dose used (Gorer and Kaliss, 1959), and about 50 per cent of the cells are killed *in vitro*. The third category includes tumours which are completely insensitive to the action of antibody, and Snell (1957) puts into this class the adenocarcinoma D1905, fibrosarcoma S620 and perhaps the adenocarcinoma C_3HBA, which was tested by Algire, Weaver and Prehn (1954) in a diffusion chamber, and the tumour B3 with which Miller (1956) failed to show a cytotoxic effect of iso-antibody in tissue culture.

Kaliss (1958) believes that immunological enhancement is due to a direct stimulant effect of antibody upon the tumour cells, so that the tumour is altered in some way and is thus better able to withstand and overcome the hosts' reaction. The present author (O'Gorman and Mikulska, 1960) by absorption experiments has demonstrated marked deficiencies of the H–2 antigens E and K from the cells of Sa1, a tumour which is very readily enhanced. Sa1 may be insensitive to cytotoxic antibody because of its antigenic deficiencies, but this need not preclude a stimulant effect of the antibody on the cell. Similar antigenic loss has not been studied with other tumours, but it is known to occur in the early transplant generations of transplantable tumours.

Gorer and his co-workers have shown that circulating antibody when passively transferred will protect the host against a graft of at least some tumours, and the

neutralization technique has been widely used to demonstrate the toxic effect of antibody upon cells which were subsequently grafted. The work which has been reported above has demonstrated that these antibodies are toxic *in vitro*. In all cases the antigenic system involved has been the H–2 system, except for Gorer and Amos' antigen X. It would now seem to be beyond doubt that circulating antibody plays a very significant role in the rejection of at least certain classes of homograft, notably the leukoses and some sarcomata, and although its role in the rejection of grafts of some normal tissues, for example skin, is admittedly a minor one, it can no longer be held that the mechanism of homograft rejection is entirely cellular, or that this major antibody has no function. The cellular factors responsible for homograft rejection may in fact be cell-bound antibodies.

SUMMARY

A method has been described for demonstrating the cytotoxic action of murine iso-antisera *in vitro*. These cytotoxic antibodies are directed against the H–2 system of antigens and two previously unknown alleles D^k and K^b have been identified. The effect of iso-antibody on normal leucocytes and several tumours has been described, and the significance of these results and their bearing on the role of circulating antibody in the homograft reaction discussed.

REFERENCES

Algire, G. H., Weaver, J. M. and Prehn, R. T.—(1954) *J. nat. Cancer, Inst.*, **15**, 493. (1957) *Ann. N.Y. Acad. Sci.*, **64**, 1009.
Amos, D. B.—(1955) *Brit. J. Cancer*, **9**, 216.
Idem and Day, E. D.—(1957) *Ann. N.Y. Acad. Sci.*, **64**, 851.
Idem and Wakefield, J. D.—(1958) *J. nat. Cancer Inst.*, **21**, 657.
Brown, G. C.—(1943) *J. Immunol.*, **46**, 319.
Gorer, P. A.—(1956) *Advanc. Cancer Res.*, **4**, 149.—(1958) *Ann. N.Y. Acad. Sci.*, **73**, 707.
Idem and Amos, D. B.—(1956) *Cancer Res.*, **16**, 338.
Idem and Kaliss, N.—(1959) *Ibid.*, **19**, 824.
Idem and Mikulska, Z. B.—(1954) *Ibid.*, **14**, 651.—(1959) *Proc. Roy. Soc.* B, **151**, 57.
Idem, Mikulska, Z. B. and O'Gorman, P.—(1959) *Immunology*, **2**, 211.
Idem and O'Gorman, P.—(1956) *Trans. Bull.*, **3**, 142.
Kaliss, N.—(1958) *Cancer Res.*, **18**, 992.
McGhee, R. B.—(1952) *Proc. Soc. exp. Biol., N.Y.*, **80**, 419.
Miller, D. G.—(1956) *J. nat. Cancer Inst.*, **16**, 1473.
Mitchison, N. A.—(1955) *Trans. Bull.*, **2**, 93.
O'Gorman, P. and Mikulska, Z. B.—(1960) *Brit. J. Cancer*, **14**, 121.
Pappenheimer, A. M.—(1917) *J. exp. Med.*, **25**, 629.
Ritz, H.—(1911) *Z. ImmunForsch.*, **9**, 321.
Schrek, R.—(1936) *Amer. J. Cancer*, **28**, 389.
Snell, G. D.—(1957) *Cancer Res.*, **17**, 2.

BILE ACID COMPOSITION AND BILE VOLUME IN BUTTER YELLOW FED RATS IN RELATION TO LIVER CANCER

S. MIRVISH AND J. GILLMAN

From the Department of Physiology and Joint C.S.I.R./University Nutrition Research Unit, University of the Witwatersrand, Johannesburg, South Africa

Received for publication April 4, 1960

PRIMARY carcinoma of the liver is a common disease amongst Africans (Berman, 1958), but the aetiology and mechanism still remain to be disclosed. Butter yellow (4-dimethylaminoazobenzene) produces liver cancer when fed to rats, and it was hoped that a better understanding of the action of butter yellow (BY) in rats would help to elucidate the mechanism of liver cancer in man. BY does not usually produce cancer in organs other than the liver, which suggests that at some stage in the chain of events initiated by BY a modification occurs in a metabolic pathway which is confined to the liver, and that the modification culminates in carcinoma. It seemed that one such pathway might be the degradation of cholesterol to bile acids, which is probably confined to the liver (Harold, Felts and Chaikoff, 1955).

The cholesterol-bile acid pathway receives added importance in view of the conversion of bile acids and cholesterol into carcinogens. The carcinogen methylcholanthrene can be synthesized chemically from deoxycholic or cholic acids (reviewed by Greenstein, 1954, p. 49), and cholesterol can be oxidized to several carcinogenic compounds (Fieser, Greene, Bischoff, Lopez and Rupp, 1955). Even deoxycholic acid itself when injected under the skin of mice can lead to sarcomas (Cook, Kennaway and Kennaway, 1940). A particular disturbance in bile acid metabolism therefore might conceivably lead to the endogenous production of a carcinogen, a possibility that has often been suspected.

A further indication for a link between cholesterol metabolism and hepatocarcinogenesis was supplied by Gillman, Gilbert and Spence (1954). These workers emphasized the frequent occurrence of bile duct hyperplasia in the livers of BY-fed rats, and they suggested an association between bile duct hyperplasia and a raised serum cholesterol, both of which were shown by these investigators to occur together after ligation of the bile duct, in hypothyroidism and (possibly) in avitaminosis A. Since then Spain and Griffin (1957) have found an increased serum cholesterol after feeding the hepatocarcinogen 3′-methyl-4-dimethylaminoazobenzene for three weeks, at which stage bile duct hyperplasia also declares itself. This rise in serum cholesterol might be linked to a fall in the rate of cholesterol degradation, which proceeds almost entirely by the pathway to bile acids (Siperstein, Jayko, Chaikoff and Dauben, 1952).

In this paper it is accordingly proposed to examine the bile acid metabolism of BY-fed rats and controls, as expressed by the amount and composition of the bile acids obtained after cannulation of the main bile duct. Details of the analytical method are supplied. Attention will be drawn to the effects of cannulation on the liver, and an assessment will be made of the influence on bile composition of the

liver damage induced by cannulation. It will then be shown that BY affects the volume of bile and the relative amounts of dihydroxy- and trihydroxycholanic acids. The significance of the latter observation will be discussed in terms of the biosynthesis of bile acids from cholesterol.

MATERIAL AND METHODS

Material

Stock male rats of the G.G. strain (Gilbert, Gillman, Loustalot and Lutz, 1958) were reared on a full basal diet (*ibid.*) to which BY (0·06 per cent of diet) was added as a 3 per cent solution in arachis oil, from the age of 3–4 months (these rats are henceforth referred to as "BY rats"). This regimen produces liver tumours in most rats of our stock after 6–8 months. At the same time control rats of the same age and sex were reared on the basal diet alone.

Collection of bile

When BY had been fed for periods varying from 2 to 5 months, the bile ducts of groups of 3–5 BY rats weighing 200–300 g. were fistularized and the bile was collected in glass saddles (van Zyl, 1957). The saddles allow the rats to move around freely in contrast to the restraining cages usually employed. With each group of BY rats a corresponding number of control rats was also fistularized.

After the operation the majority of the rats secreted bile copiously for several days at least. They were maintained on the same diet except that 0·85 per cent NaCl was supplied instead of water. A preparation containing 10,000 i.u. penicillin and 50 mg. streptomycin was injected subcutaneously after operation and on the subsequent two days.

Preliminary experiments showed that the presence of thymol did not interfere with the bile acid analyses. Accordingly 0·2 ml. of 5 per cent thymol in ethanol was added every evening as preservative to each saddle. The bile was collected from the saddles twice daily and where necessary was stored at -15° C. For most of the experiments the bile from each rat was pooled from the second to the fourth days after cannulation. Where the bile from all three days was not available due to spillage, blockage of the cannula or other accidents, the bile was pooled from as many of these days as possible. Bile secreted on the day before death was discarded. Some rats died after a varying period of bile drainage and some were killed with ether, as detailed later. After death the rats were examined and the livers were preserved in formalin. Sections for histological examination were taken from at least the left lateral, left median and right median lobes.

Analysis of the bile acids

The bile acids were extracted by a modification of the method of Mosbach, Kalinsky, Halpern and Kendall (1954) from three aliquots of each bile sample, each aliquot consisting of $\frac{1}{3}$–$\frac{1}{2}$ of the mean daily production of bile, corresponding to 4–8 ml. bile and 5–20 mg. bile acids. Each aliquot was heated with NaOH (final concentration 1·5 N) in a sealed pyrex test-tube at 120° for 4 hours to hydrolyze the conjugated bile acids. The hydrolysate was extracted with petrol ether (40–60°; 2 × 20 ml.), which was then re-extracted with water (2 × 10 ml.). The combined aqueous phase was acidified with HCl and extracted with re-distilled

ether (5 × 20 ml.). The ether extract was shaken with water (5 × 5 ml.) to remove the HCl, dried over sodium sulphate, evaporated and stored over P_2O_5 in a dessicator. Storage for several months did not affect the subsequent analyses.

The trihydroxycholanic acids (THA) and dihydroxycholanic acids (DHA) in the samples were analysed by quantitative, reversed phase, partition chromatography on columns of non-wetting kieselguhr. The method employed was an extension of the qualitative method of Bergström and Sjövall (1951) and Sjövall (1953) and has been briefly described in previous publications (Mirvish, 1957, 1958). A 1 ml. siphon, a mixing vessel and 0·01 N methanolic NaOH in a burette were used to titrate each 1 ml. fraction as soon as it was eluted. Each titration was carried out to an end-point of pH 8·8 using a Beckman Model G pH meter, as recommended by Dr. A. Antonis (private communication). The meter was connected by shielded wire to a glass electrode and a calomel half-cell in which was inserted a bridge of agar-saturated KCl. The glass electrode and agar bridge dipped into the mixing vessel through side arms sealed with rubber tubing. The nitrogen was passed through soda lime before entering the mixing vessel.

The solvents for chromatograpy were freshly mixed each day and a trace of HCl (0·0002 N) was added to the moving phase to prevent losses on the column. The acids were applied as a solution in moving phase (5 ml.) immediately after 50 ml. of moving phase had been run through the column. The flow rate was adjusted to 1 ml. per 90–100 seconds. A " base line " figure of 0·015–0·030 ml., which corresponded to the added HCl and was best determined after elution of all the bile acids, was subtracted from the results of each titration. Controls were run at frequent intervals with mixtures of pure cholic and deoxycholic acids, by simple chromatography and by chromatography following alkali treatment and extraction.

Two of the three aliquots from each bile sample were chromatographed and, if the results differed by more than 2·0 mg. for THA (total 10–20 mg.) and 0·5 mg. for DHA (total 0–4 mg.), then the third aliquot was also chromatographed. The results for THA and DHA were expressed as concentrations or as mg./24 hours/100 g. body weight, as calculated from the mean titration figure, the bile volume and the mean weight of the rat on the days during which the bile was secreted. The bile volume was expressed similarly as ml./24 hours/100 g. body weight.

Accuracy of the analytical method

Mixtures of 10 mg. cholic acid and 2 mg. deoxycholic acid were eluted from the columns with recoveries of 95 ± 3 per cent and 119 ± 10 per cent respectively (A ± B signifies mean and standard deviation throughout this paper). When similar amounts of the acids were added to bile samples before alkali treatment, the acids were recovered in yields of 80–110 per cent (Mirvish, 1958).

The accuracy of the complete extraction and estimation was tested by analysis of the difference between the duplicate values obtained in the present investigation. The standard deviation (S) was calculated for the error of the average of two determinations on the same sample using the formula $S^2 = \Sigma(x_1 - x_2)^2/4n$, where x_1 and x_2 are the two values and n is the number of samples. The results were 1·0 mg. for THA (control and BY rats), 0·15 mg. for DHA (controls) and 0·27 mg. for DHA (BY rats). These values should be compared with the average figures for all results (11·6, 1·39 and 1·20 mg. respectively—see Table I) and are in the same units, namely mg./24 hours/100 g. body weight.

RESULTS

Time of Survival and Loss of Weight after Cannulation

Six control rats died within the first six days after cannulation, nine died on days 7–9, and the remaining 24 controls were killed with ether ; 13 on days 5–10, nine on days 11–13 and two on days 19–20. In contrast, 16 of the 34 BY rats died within the first six days after cannulation, eight died on days 7–10 and three on days 16–20. In addition, seven BY rats were killed with ether on days 5–6. It is clear from these results that feeding BY shortens the time of survival after cannulation of the bile duct.

The BY rats lost more weight between cannulation and death than did the controls. For example, the four BY rats which survived the operation for 10 days or more each lost 92–121 g. after cannulation, whereas no control rat lost more than 60 g. The BY rats lost weight steadily at a rate of 4–8 g./day throughout the period of bile drainage ; the controls lost weight at a similar rate until the fourth day, but thereafter the rate fell to 1·5–3·0 g./day.

Changes in Bile Composition Induced by Butter Yellow

In order to separate effects on bile acid secretion from effects on bile volume, the results are mostly expressed in terms of the secretion per 24 hours per 100 g. body weight, rather than as concentrations.

Daily variations in bile composition

Analyses were carried out on the bile samples secreted each 24 hours by six control and eight BY rats (Fig. 1). The THA, DHA and bile volume fell in both groups of rats on the second day after cannulation, rose to a maximum on the fourth to sixth day and thereafter decreased at varying rates. The values for THA and bile volume were similar in BY and control groups. On the first day after cannulation the proportion of DHA to THA (Fig. 1*d*) in the BY group was as high as that in the control group, but thereafter the BY figure remained consistently below that for the controls, suggesting that differences in DHA might exist between the two groups of rats after the first day.

The correlation coefficient for the relation between THA and DHA in the daily samples was calculated separately for each rat. In the control group two rats showed no significant correlation, three a 90 per cent and one a 99 per cent correlation. In the BY group two rats showed no correlation, one a 90, one a 95, one a 98, two a 99 and one a 99·9 per cent correlation. The correlations are thus much stronger in the BY group than in the control group, but insufficient data were available to test rigidly the significance of this difference between the two groups of rats.

Bile composition of samples pooled over several days

In view of the results of the daily analyses, in subsequent experiments the bile secreted by each rat during the second, third and fourth days after cannulation was pooled before investigation, in order to reduce the number of rather tedious analyses. Thirty-four BY and 39 control rats were examined in this way, including eight BY and six control rats where the mean bile composition for the three days

was calculated from the daily analyses. The values obtained for THA, DHA ad bile volumes are summarized in Table I. The relationship between THA and DHA is shown for each rat in Fig. 2 and 3. For reasons which will become apparent

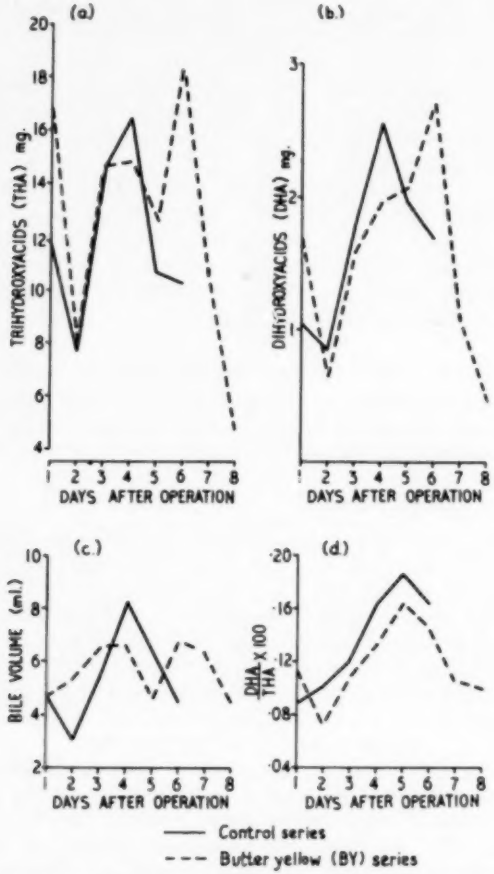


FIG. 1.—Daily variations in bile composition. Trihydroxyacids (THA), dihydroxyacids (DHA) and bile volume are expressed in mg. or ml./24 hours/100 g. body weight. The curves represent average figures for six control and for eight butter yellow rats.

below, the results in Table I are shown not only for all BY and all control rats, but also separately for those rats of each group with a THA exceeding 10 mg./day/100 g. and for those with less than 10 mg. THA.

Three main conclusions can be drawn from Table I.

Firstly, though there was a wide scatter of THA from rat to rat, there was no significant difference in THA distribution between BY and control rats. As THA

is by far the major fraction of the bile acids, it is concluded that BY did not significantly affect the total secretion of bile acids.

Secondly, BY affected the production of DHA under certain defined conditions. If all the results are taken into account irrespective of the amount of THA secreted, the mean DHA is not significantly different in the BY and control groups, though

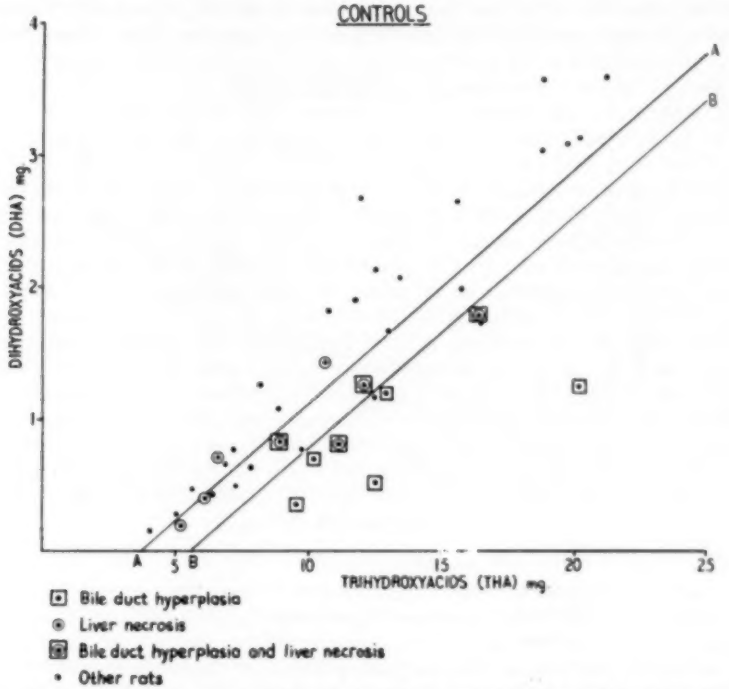


FIG. 2.—Relationship between trihydroxyacids (THA (and dihydroxyacids (DHA) in the control series, for bile pooled on the second to fourth days after cannulation. THA and DHA are expressed in mg./24 hours/100 g. body weight. AA = regression line (DHA = 0·176 × THA − 0·65). The nine points below BB constitute the "low DHA group". Points for rats with *any* degree of bile duct hyperplasia and/or necrosis are differentiated from points for rats with relatively normal livers.

TABLE I.—*Trihydroxyacids (THA), Dihydroxyacids (DHA) and Bile Volume in Control Rats and Rats Fed Butter Yellow, for Bile Pooled on the Second to Fourth Days After Cannulation*

	Total		THA less than 10 mg.		THA more than 10 mg.	
	Control	BY	Control	BY	Control	BY
Number of rats	39	34	16	10	23	24
THA (mg.)	11·6±4·75	12·4±4·04	7·2±1·65	7·4±1·63	14·7±3·56	14·5±2·58
DHA (mg.)	1·39±0·99	1·20±0·61	0·58±0·38	0·57±0·47	1·96±0·90	1·47±0·44
Bile volume (ml.)	5·2±1·40	6·20±1·58	4·5±1·02	5·1±1·45	5·7±1·46	6·7±1·37

the variability of DHA is definitely reduced in the BY rats (98 per cent level). However, if only results from rats secreting more than 10 mg. THA are considered, then the mean value for DHA is significantly lower (95 per cent level) in the BY group. No BY rat secreted more than 2·2 mg. DHA, though the DHA of seven of the 39 controls exceeded this value. BY thus caused a reduction in DHA, but only when a comparatively large amount of THA was produced.

Thirdly, the daily volume of bile was greater in the BY rats than in the normals when all rats were considered irrespective of the value for THA (99 per cent level

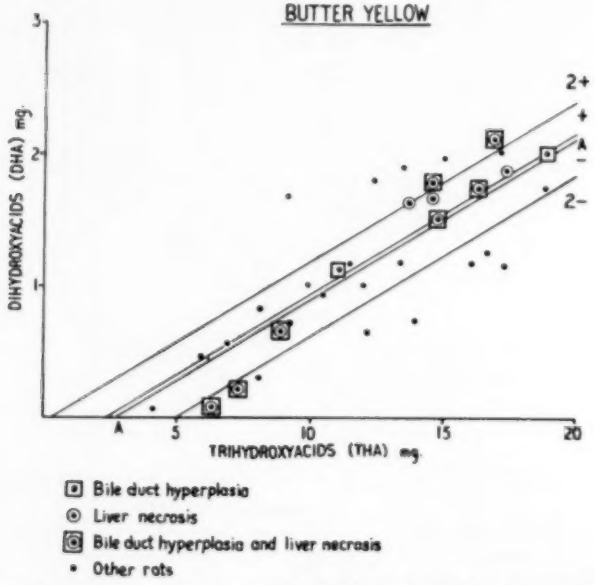


FIG. 3.—Relationship between trihydroxyacids (THA) and dihydroxyacids (DHA) in the butter yellow series, for bile pooled on the second to fourth days after cannulation. THA and DHA are expressed in mg./24 hours/100 g. body weight. AA = regression line (DHA = 0·122 × THA − 0·32). The rats are graded according to the position of their points relative to the lines, as indicated by the symbols 2+ to 2− on the right of the figure. Points for rats with *severe* bile duct hyperplasia (3+ grading) and/or *severe* liver necrosis (2+ grading) are distinguished from points for rats with milder liver damage.

of significance), and when only those rats were considered with more than 10 mg. THA (95 per cent level). No significant differences in volume were found for rats with less than 10 mg. THA.

BY feeding thus led to a decrease in the secretion of DHA for the group with more than 10 mg. THA, and to an increase in bile volume. BY should therefore also produce a decrease in the *concentration* of DHA (daily amount of DHA divided by daily volume). No significant decrease in the concentration of DHA is noted if all results are considered. If only those rats secreting more than 10 mg. THA/day/100 g. are taken into account, the concentration of DHA is 38·1 ± 22·3 mg./100 ml. bile in the controls and 22·5 ± 7·94 mg./100 ml. in the BY group

(significantly different at the 99 per cent level); the corresponding concentrations of THA are 276 ± 90 and 223 ± 98 mg./100 ml. (no significant difference).

Both BY and control groups showed significant correlations between THA and DHA (99·9 per cent level) and between THA and volume (99 per cent level). In addition, the BY group showed a striking correlation (99·9 per cent level) between DHA and bile volume, though the control group here showed no correlation at all.

The first order regression lines were calculated for DHA as a function of THA in the BY and control groups (Fig. 2 and 3) and for DHA as a function of volume in the BY group. The regression lines for the DHA/THA diagrams do not pass through the origin, so that little or no DHA is produced when THA falls below 3–4 mg. As the amount of THA rises above this figure, the proportion of DHA to THA increases rapidly, especially in the control series. The control rats with a very high THA seem to show a DHA even greater than that expected from the calculated regression line, suggesting that the true regression line may here be of the second order. These observations demonstrate the importance of determining total daily secretions and not merely the ratios between the different bile acids.

The changes in bile composition induced by BY may be summarized as follows: (1) DHA (expressed either as daily secretion or as concentration) was decreased in those cases where the daily secretion of THA exceeded 10 mg./100 g. body weight. This decrease was associated with a decrease in the inter-individual variability of DHA. (2) Bile volume was increased. (3) A strong inter-individual correlation developed between DHA and volume (for the pooled samples from different rats), and (4) the intra-individual correlation between DHA and THA was probably increased (for different daily samples from a single rat). It should be emphasized that BY did not affect the production of THA, and that the DHA/THA ratio rose with an increase in THA, especially in the control series.

Factors Affecting the Inter-individual Variations in Bile Composition

An investigation of the factors responsible for the considerable overlap between the results of the BY and control groups might increase the significance of the difference between the two groups of rats. The livers showed variable degrees of acute necrosis and bile duct hyperplasia in both series of rats, so that some of the variations in bile composition might be explained in terms of the extent of liver damage. In the following sections, the interrelationships will be examined between bile composition, liver histology and the reaction of the rat to cannulation.

A. *Relationship between bile composition and liver damage in the control rats*

Eighteen of the 31 livers examined from the control series appeared almost normal, and 13 showed some degree of bile duct hyperplasia and/or necrosis. The extent of each form of liver injury was graded 0, ±, +, 2+ or 3+. A variable degree of bile duct hyperplasia was observed in nine rats (± in four, + in one and 2+ to 3+ in four). A mild necrosis was observed in eight rats (± in five, + in two and 2+ in one). These figures include those from four rats with both types of injury. As rats of the same strain do not normally show spontaneous liver damage, much of the observed injury was probably a consequence of the cannulation and subsequent bile drainage.

Three of the rats with bile duct hyperplasia had been killed on the fifth day after cannulation, two had died on the seventh day and four had been killed after

8–13 days. Thus in at least some instances bile duct hyperplasia was present at the time when the bile was being collected for analysis, i.e. on the second to fourth day after cannulation, and any association found between bile composition and this form of liver damage is probably direct.

Two of the control rats with necrosis had died on the fifth day after cannulation, three had died or had been killed on days 7–8 and three had been killed on days 11–13. It is thus not clear whether the necrosis developed during the period when the bile was being collected for analysis or later, and the significance of any relationship found between necrosis and bile composition will therefore be obscure.

Nine of the 39 control rats showed points on the DHA/THA graph below line BB (Fig. 2) and were hence deemed to have secreted an unusually low DHA relative to THA. This low DHA group contained seven of the nine rats with bile duct hyperplasia (Fig. 2); the liver of one of the remaining members of the low DHA group was normal and the other liver was lost. The mean bile volume was 6·56 ml./day/100 g. body weight for the nine rats with bile duct hyperplasia, compared with 5·20 ml. for all the controls and 6·20 ml. for the BY rats. Bile duct hyperplasia due to cannulation of the bile duct in normal rats apparently affects bile composition in the same way as does BY administration, namely by lowering DHA relative to THA and increasing the bile volume. These changes in bile composition are, therefore, not specific to the process of BY carcinogenesis.

For the 16 control rats which did not show bile duct hyperplasia and which secreted more than 10 mg. THA, the DHA was 2·35 $\pm$ 0·76 mg./day/100 g. as compared with 1·96 $\pm$ 0·90 mg. for all control rats with more than 10 mg. THA and 1·47 $\pm$ 0·44 mg. for the BY rats with more than 10 mg. THA (Table I). Thus exclusion of the control rats with bile duct hyperplasia accentuates the difference in mean DHA between control and BY groups. The difference is now significant at the 99·9 per cent level instead of the 95 per cent level found when all control rats were considered, irrespective of the histology of their livers. Furthermore, the variability in DHA of the control group has been reduced and is now close to that of the BY group.

Cannulation of the bile duct thus led in nine control rats to bile duct hyperplasia. The latter was associated with a bile composition similar to that of the BY rats. These changes in bile composition are therefore not specific. If the results from the control rats with bile duct hyperplasia are excluded, then the difference in mean DHA between BY and control groups is greatly accentuated.

B. *Relationships between bile composition, liver damage and reaction to cannulation in the BY rats*

Two factors, which do not arise in the control series, need to be taken into account when investigating the BY series. Firstly, in addition to cholangiofibrosis and cancer, BY by itself produces bile duct hyperplasia and necrosis of the liver, which closely resemble the similar reactions resulting from cannulation. Secondly, with regard to bile composition it was only necessary in the control series to consider the correlation between DHA and THA; in the BY series the correlation between DHA and volume, which is absent in the controls, also has to be taken into account.

In view of these complications, a system was adopted whereby the performance of each BY rat was graded with respect to each of four factors: (1) the relation

of DHA to THA, (2) the relation of DHA to bile volume, (3) the extent of bile duct hyperplasia and (4) the extent of liver necrosis.

A grading was assigned to each rat according to the position of its point on the DHA/THA graph relative to the regression line, positive gradings signifying a relatively high DHA and negative gradings a low DHA (Fig. 3). A second grading was assigned similarly from a graph of DHA plotted against bile volume. The two gradings were of opposite sign for four rats; three of these produced less than 8·5 mg. THA and the fourth produced 10·0 mg. THA. For eight rats one or both of the points lay very close to the regression line (i.e. with grading ±). For the remaining 22 rats the two gradings were both definitely positive or both definitely negative. Thus DHA tended either to be high relative to both THA and bile volume, or to be low relative to both these factors, except where the total production of bile acids was very low.

In order to avoid dealing with gradings for both relationships (DHA/THA and DHA/Volume), the two gradings were added to give a composite "bile grading", except in the four cases with gradings of opposite sign, where no "bile grading" was assigned. This composite grading will be used in the ensuing discussion as a general index of the extent to which bile composition departs from the normal. A high bile grading (up to 4+) indicates a relatively normal bile composition and a low bile grading (down to 4−) indicates a relatively abnormal bile composition.

Necrosis was present in 27 of the 33 livers examined and bile hyperplasia in 22. Gradings from 0 to 3+ were assigned for each type of liver damage, as with the control rats. Five livers showed cholangiofibrosis and/or small cancerous foci.

We may now attempt to determine whether variations in the bile composition of BY rats are associated with variations in the reaction of the liver to cannulation, as in the case of the control rats. In order to facilitate analysis, the data were grouped according to the values for bile grading, days lived after cannulation and length of time for which BY was fed (Tables II, III and IV). The seven cases with less than 8·5 mg. THA, together with the rat which secreted 10·0 mg. THA but showed gradings for DHA/THA and for DHA/Volume of opposite sign, are listed as a separate "low bile acid group" in Table II and are omitted from consideration in Tables III and IV.

The bile gradings for the five rats with cancerous nodules and/or cholangiofibrosis varied widely, so that neither cancer nor cholangiofibrosis appears to affect bile composition, apart from the general effects of BY.

The eight rats of the low bile acid group showed definite liver necrosis (with one exception) and a variable degree of bile duct hyperplasia (Table II). All but

TABLE II.—*Extent of Liver Damage in the Butter Yellow Rats, Grouped According to Bile Grading*

Bile grading	Number of rats	Degree of necrosis				Degree of bile duct hyperplasia				
		3+	1+	±	0	3+	2+	1+	±	0
3+ to 4+	9	5	3	1	—	3	1	2	1	2
± to 2+	7	2	2	2	1	3	2	1	—	1
1− to 4−	10	—	3	3	4	1	—	3	2	4
*Low bile acid	8	2	3	1	1	2	—	1	1	3
All values	34	9	11	7	6	9	3	7	4	10

* One liver was lost here.

one of these rats died within six days of the cannulation. Total or partial obstruction of the bile flow leads within 48 hours to liver necrosis and bile duct hyperplasia (Cameron and Oakley, 1932; Gillman, Gilbert and Spence, 1954), so that biliary obstruction was probably the main cause in this group of the low production of bile acids, the necrosis and the early death. These cases are not considered in the following two paragraphs.

Nineteen rats were not deliberately killed and did not belong to the low bile acid group. Of these, all ten rats living for seven or more days after cannulation showed positive bile gradings, including seven out of the nine rats with bile gradings of 3+ or 4+ (Table III). The five rats with negative bile gradings lived for

TABLE III.—*Bile Gradings and Extent of Liver Damage in the Butter Yellow Rats, Grouped According to Manner of Death and Days Lived After Cannulation*

Manner of death	Time of survival after operation (days)	Number of rats	Bile grading				Necrosis				Bile duct hyperplasia				
			3+ to 4+	± to 2+	1− to 2−	3− to 4−	2+	1+	±	0	3+	2+	1+	±	0
Died	3–6	9	2	2	5	—	1	4	2	2	1	—	4	—	4
Died	7–20	10	7	3	—	—	6	1	2	1	5	1	2	1	1
Killed with ether	5–6	7	—	2	1	4	—	3	2	2	1	2	—	2	2

less than six days. Cannulation thus precipitates death least rapidly in those rats with the most positive bile gradings, i.e. in those rats with bile compositions which approach closest to the normal.

The maximum liver damage was found in rats with high bile gradings, i.e. with relatively high values for DHA (Table II, Fig. 3), in contrast with the exactly opposite position found for the control rats (Fig. 2). The BY rats thus showed a paradoxical association of liver damage with a relatively normal bile composition. The explanation of the paradox is clearly that the BY rats with high bile gradings survived the longest after cannulation (preceding paragraph) and so were more likely to develop liver injury. Thus 2+ necrosis and/or 3+ bile duct hyperplasia were found in seven of the ten rats living for seven or more days after cannulation, but in only three of the 16 rats which died or were killed before the seventh day (Table III). The extent of liver injury was much greater than that expected in non-cannulated BY rats, and it is clear that most of the injury observed in the BY rats developed as a response to prolonged bile drainage. In the control rats liver damage was far less severe, even in those rate surviving for a considerable period after cannulation. BY thus increased the vulnerability of the liver to prolonged bile drainage. This increase in vulnerability was possibly responsible for the BY rats dying sooner after cannulation than the controls.

A correlation has clearly not been established in the BY rats between bile composition and the condition of the liver *at the time when the bile was being collected for analysis*, i.e. on the second to fourth days after cannulation. Bile composition is less variable in the BY rats then in the controls, and so is apparently not as easily affected by differing responses to cannulation.

The extent of liver necrosis and of bile duct hyperplasia tended to increase as the period of BY feeding was lengthened from 63 to 153 days (Table IV). The mean bile gradings, however, did not alter significantly during this period (Table IV). The reduction in DHA from the normal values had thus occurred to the

TABLE IV.—*Bile Gradings and Extent of Liver Damage in the Butter Yellow Rats, Grouped According to the Length of Time for which Butter Yellow was Fed*

Days on butter yellow	Number of rats	Bile grading				Necrosis				Bile duct hyperplasia				
		3+ to 4+	± to 2+	1− to 2−	3− to 4−	2+	1+	±	0	3+	2+	1+	±	0
63–81	6	2	1	3	—	1	—	3	2	—	—	3	—	3
98–118	12	3	7	1	1	4	4	2	2	6	2	1	1	2
138–153	8	4	—	1	3	2	4	1	1	1	1	2	2	2

same extent after 63–81 as after 138–153 days of BY feeding, and probably took place within the first 60 days.

From the foregoing it would appear that : (1) Cases with a high DHA relative to THA also show a high DHA relative to bile volume. (2) The presence of cancerous nodules or cholangiofibrosis does not affect bile composition, apart from the general effects of BY. (3) Eight rats show a low secretion of total bile acids, associated with extensive necrosis and early death after cannulation. (4) Cannulation leads to death more rapidly in those BY rats having the more abnormal bile compositions. (5) BY rats are vulnerable to the effect of bile drainage and usually develop extensive liver damage about seven days after cannulation. (6) Bile composition is probably affected within 60 days of BY feeding.

DISCUSSION

The findings will now be discussed with special reference to the effects of cannulating the bile duct, cholesterol metabolism, and conditions other than BY treatment which are known to affect bile acid metabolism. A mechanism involving changes in the rate of 12-hydroxylation will be proposed to explain observed changes in DHA/THA ratios, and the possible significance will be examined of hydroxylation in general for the understanding of cancer.

Death follows cannulation of the bile duct more rapidly in BY rats than in the controls, and more rapidly in BY rats with low bile gradings than in BY rats with high bile gradings. The rats could have died from a deficiency of a fat-soluble vitamin, e.g. vitamin K (some rats bled very easily after a few days of bile drainage and a deficiency of vitamin K is well known to result after the bile duct has been fistularized in man and in the dog).

The daily production of bile acids in the intact rat is only about 2 mg./100 g. body weight (Linstedt and Samuelsson, 1959) as compared with the 10–20 mg./100 g. found for cannulated rats in the present and previous investigations (Eriksson, 1957). Thus bile acid production is greatly increased by cannulation of the bile duct. In a rat with an intact entero-hepatic circulation the bile acids returning in the portal vein probably inhibit the further production of bile acids by the liver (Bergström and Danielsson, 1958). Now the bile of BY rats secreted on the first day after cannulation appears to show a normal DHA/THA ratio (Fig. 1*d*). As this bile largely represents bile that had been circulating in the intact rat, it seems likely that BY does not reduce DHA in the intact rat. BY has thus been demonstrated to lower DHA only when the production of bile acids is strained to a maximum due to the cannulation (and even then the effect is only noted when bile acid production rises above 10 mg./100 g.).

BY did not affect the total production of bile acids in the cannulated rat. This result does not support the view that the raised serum cholesterol which is apparently associated with bile duct hyperplasia (Gillman, Gilbert and Spence, 1954), is due to a decreased degradation of cholesterol to the bile acids.

Eriksson (1957) showed that the DHA (actually chenodeoxycholic acid was measured) rose from the normal 10–20 per cent of the total bile acids to 50–60 per cent on feeding desiccated thyroid and fell to 5 per cent on feeding thiouracil. The action of BY on DHA could therefore be due to a depression of thyroid function. The latter is not known to be affected by BY, though thiouracil delays the production of hepatomas by BY (Paschkis, Cantarow and Stasney, 1948; Harris and Clowes, 1952).

Carey (1958), Rudman and Kendall (1957) and Osborn, Wootton, da Silva and Sherlock (1959) have recently reported on the levels of serum bile acids in various types of jaundice. Though the results from the three groups of workers differ considerably, it is agreed that the THA/DHA ratio is almost always below 1·0 in cases of portal cirrhosis, and is above this figure in obstructive jaundice. Carey found that six of the seven cases of obstructive jaundice due to carcinoma (of various types) showed THA/DHA ratios greater than 3·8, compared with two of the ten patients with other types of obstructive jaundice. Thus DHA production may be relatively depressed in human cancer, reminiscent of the position in the BY rats. The cancer cases examined by Osborn *et al.* showed no depression of DHA.

If one may speculate, the level of DHA relative to that of THA could serve as a useful indicator of liver metabolism in various diseases, especially if cirrhosis on the one hand and bile duct hyperplasia and liver cancer on the other hand are found to affect the proportion of DHA in opposite directions. It is possible that conditions only become favourable for the development of liver cancer when DHA secretion is diminished. The bile acids may themselves influence liver structure and function on their return to this organ via the portal vein; the bile acids returning to the liver are already known to regulate the further synthesis of bile acids. Finally, it should not be forgotten that changes in bile acid composition could also affect the digestion and absorption of lipids.

Possible mechanism for changes in the relative amounts of DHA and THA

Cholic acid (3 : 7 : 12-trihydroxycholanic acid) is the main acid of the THA fraction, apart from minor amounts of 3 : 6 : 7-trihydroxycholanic acids (Hsia, Matschiner, Mahowald, Elliott, Doisy, Thayer and Doisy, 1957, 1958). Chenodeoxycholic acid (3 : 7-dihydroxycholanic acid) is the main acid of the DHA fraction (Bergström and Sjövall, 1954). The depressed DHA in the BY rats is thus due, presumably, to changes in the relative rates of synthesis of cholic and chenodeoxycholic acids. According to Bergström (1959), these syntheses proceed as follows: The first step for both acids is the hydroxylation of cholesterol to form 7α-hydroxycholesterol. In the case of chenodeoxycholic acid the 3-hydroxy group is then converted from the β to the α configuration, the double bond at C_5–C_6 is saturated and finally the side chain is oxidized with the removal of three carbon atoms. The identical changes take place during the formation of cholic acid, except that a 12α-hydroxy group is inserted at some stage before side-chain oxidation. Chenodeoxycholic acid is not directly converted into cholic acid.

It appears likely that the relative amounts of DHA and THA are controlled, in part at least, by the rate of 12-hydroxylation, as this reaction constitutes the only difference between the syntheses of cholic and chenodeoxycholic acids. In the present paper the production of DHA relative to that of THA has been modified in three circumstances: (1) Feeding BY reduces the DHA (at high levels of THA). (2) The proportion of DHA rises as the total production of bile acids rises, especially in the control series. (3) In BY rats the amount of DHA rises with an increase in bile volume.

If the effects are due in all three circumstances to modifications in the rate of 12-hydroxylation, it follows that: (1) feeding BY leads to an acceleration of 12-hydroxylation, (2) as more bile acids are formed, the enzymes controlling 12-hydroxylation can cope with a smaller proportion of the bile acids, so that more DHA is formed, and (3) in BY rats secreting a large volume of bile, the proportion of bile acids which escapes 12-hydroxylation is increased, possibly because the bile is swept out of the liver lobule before hydroxylation can be completed. For unknown reasons the last factor does not operate in normal rats. It has also been mentioned that there is an increased formation of DHA in hyperthyroidism and in liver cirrhosis. This increase could also be due to a decrease in the rate of 12-hydroxylation, as already proposed by Carey (1958) in the case of cirrhosis.

It will be recalled that on cannulation of the bile duct the liver is stimulated to produce an abnormally large amount of bile acids. This load which has been placed on the liver by cannulation has, according to the above hypothesis, disclosed a slight defect in the capacity of the liver to hydroxylate bile acids, which might otherwise have escaped detection.

The relation between cancer and hydroxylations in the liver

An increased rate of hydroxylation in the liver may not be confined to the possible acceleration of 12-hydroxylation by BY. Thus the intraperitoneal injection of benzpyrene, methylcholanthrene and other polycyclic hydrocarbons (carcinogenic and non-carcinogenic) leads to an increase in liver benzpyrene hydroxylase (Conney, Miller and Miller, 1957). Administration of the carcinogen acetylaminofluorene leads to cancer of the urinary bladder if tryptophan is also fed (Dunning, Curtis and Maun, 1950). Now 3-hydroxyanthranilic acid and 3-hydroxykynurenine are compounds which are carcinogenic to the bladder (Allen, Boyland, Dukes, Horning and Watson, 1957) and are normally formed from tryptophan in the liver by a series of reactions including a hydroxylation, and it seems that acetylaminofluorene might act by accelerating the formation of these two compounds by the liver.

Another connection between liver hydroxylation and cancer is that the liver can hydroxylate several aromatic amines in the ortho position to form very active carcinogens, as proposed by Clayson (1953). The hydroxylations involved in the conversions of tryptophan discussed above are of this type. Amines such as β-naphthylamine are similarly converted into carcinogenic *o*-hydroxy derivatives (Boyland, 1958). Acetylaminofluorene is converted by the liver into *o*-hydroxy derivatives which may be the active agents responsible for cancer of the liver due to this carcinogen (Weisburger, Weisburger and Morrix, 1957).

Cancer and hydroxylations may thus be linked in two ways: (1) Following Clayson's hypothesis, the liver hydroxylates amines to form carcinogenic *o*-

hydroxyamines, and (2) the rate of certain hydroxylations in the liver may be increased during the stage of induction of cancer.

SUMMARY

Details are described for a method of estimating the trihydroxy-acids (THA) and dihydroxyacids (DHA) in rat bile, using reversed phase partition chromatography. The method was applied to the analysis of samples of rat bile, collected on the second to fourth days after cannulation of the bile duct from 39 control rats and 34 rats fed butter yellow (BY) for 63–153 days.

After cannulation the BY rats died sooner and lost weight faster than the controls. The cannulation and subsequent bile drainage gave rise in many rats of both series to bile duct hyperplasia and necrosis of the liver. The BY rats were very vulnerable to the effects of bile drainage and usually developed extensive liver damage about seven days after cannulation.

Although the secretion of THA was similar in both groups of rats, the BY rats showed a decrease in DHA for those rats with a daily secretion of THA exceeding 10 mg./100 g. body weight. The BY rats also showed an increase in bile volume, and a strong correlation between DHA and bile volume which was entirely absent in the controls. The proportion of DHA to THA rose as THA secretion was increased, especially in the control series. In the control series the nine rats with bile duct hyperplasia showed changes in bile composition characteristic of those induced by BY. Exclusion of the results from these rats greatly accentuated the difference in DHA between control and BY rats. In the BY series cannulation was followed by death quickest in those rats with the most abnormal bile composition.

The significance of the depression in DHA by BY feeding is discussed in terms of other conditions which affect bile acid ratios. A mechanism involving alterations in the rate of 12-hydroxylation is proposed to explain the effect of BY feeding on DHA, the increase in the proportion of DHA with a rise in THA, and the correlation in the BY rats between DHA and bile volume. The possible significance is examined of hydroxylations in general for the understanding of cancer.

Thanks are expressed to N. G. N. Matthews and J. Makunga for excellent technical assistance and to Miss J. van Veen for carrying out the cannulations. Mr. W. Lutz very kindly carried out the statistical analysis of the data. The study was greatly facilitated by a grant made to one of us (S. M.) by the National Cancer Association of South Africa, to which body grateful acknowledgment is made.

REFERENCES

Allen, M. J., Boyland, E., Dukes, C. E., Horning, E. S. and Watson, J. G.—(1957) *Brit. J. Cancer*, **11**, 212.

Bergström, S.—(1959) 'The Biosynthesis of Terpenes and Sterols'. A Ciba Foundation Symposium. London (J. A. Churchill & Co.) p. 185.

Idem and Danielsson, H.—(1958) *Acta physiol. scand.*, **43**, 1.

Idem and Sjövall, J.—(1951) *Acta chem. scand.*, **5**, 1267.—(1954) *Ibid.*, **8**, 611.

Berman, C.—(1958) *Advanc. Cancer Res.*, **5**, 56.

Booth, J. and Boyland, E.—(1957) *Biochem. J.*, **66**, 73.

Boyland, E.—(1958) *Brit. med. Bull.*, **14**, 153.

CAMERON, G. R. AND OAKLEY, C. L.—(1932) *J. Path. Bact.*, **35**, 769.
CAREY, J. B.—(1958) *J. clin. Invest.*, **37**, 1494.
CLAYSON, D. B.—(1953) *Brit. J. Cancer*, **7**, 460.
CONNEY, A. H., MILLER, E. C. AND MILLER, J. A.—(1957) *J. biol. Chem.*, **228**, 753.
COOK, J. W., KENNAWAY, E. L. AND KENNAWAY, N. M.—(1940) *Nature, Lond.*, **145**, 627.
DUNNING, W. F., CURTIS, M. R. AND MAUN, M. E.—(1950) *Cancer Res.*, **10**, 454.
ERIKSSON, S.—(1957) *Proc. Soc. exp. Biol. N.Y.*, **94**, 578, 582.
FIESER, L. F., GREENE, T. W., BISCHOFF, F., LOPEZ, G. AND RUPP, J. J.—(1955) *J. Amer. chem. Soc.*, **77**, 3928.
GILBERT, C., GILLMAN, J., LOUSTALOT, P. AND LUTZ, W.—(1958) *Brit. J. Cancer*, **12**, 565.
GILLMAN, J., GILBERT, C. AND SPENCE, L.—(1954) *Cancer*, **7**, 1109.
GREENSTEIN, J. P.—(1954) ' The Biochemistry of Cancer '. New York (Academic Press).
HAROLD, F. M., FELTZ, J. M. AND CHAIKOFF, I. L.—(1955) *Amer. J. Physiol.*, **183**, 459.
HARRIS, P. N. AND CLOWES, G. H. A.—(1952) *Cancer Res.*, **12**, 471.
HSIA, S. L., MATSCHINER, J. T., MAHOWALD, T. A., ELLIOTT, W. A., DOISY, E. A., Jr., THAYER, S. A. AND DOISY, E. A.—(1957) *J. biol. Chem.*, **225**, 811 ; **226**, 667.—(1958) *Ibid.*, **230**, 573.
LINSTEDT, S. AND SAMUELSSON, B.—(1959) *Ibid.*, **234**, 2026.
MIRVISH, S.—(1957) *S.Afr. J. med. Sci.*, **22**, 158.—(1958) *Ibid.*, **23**, 33.
MOSBACH, E. S., KALINSKY, H. J., HALPERN, E. AND KENDALL, F. E.—(1954) *Arch. Biochem. Biophys.*, **51**, 402.
OSBORN, E. C., WOOTTON, I. D. P., DA SILVA, L. C. AND SHERLOCK, S.—(1959) *Lancet*, ii, 1049.
PASCHKIS, K. E., CANTAROW, A. AND STASNEY, J.—(1948) *Cancer Res.*, **8**, 257.
RUDMAN, D. AND KENDALL, F. E.—(1957) *J. clin. Invest.*, **36**, 530.
SIPERSTEIN, M. D., JAYKO, M. E., CHAIKOFF, I. L. AND DAUBEN, W. G.—(1952) *Proc. Soc. exp. Biol. N.Y.*, **81**, 720.
SJÖVALL, J.—(1953) *Acta physiol. scand.*, **29**, 232.
SPAIN, J. D. AND GRIFFIN, A. C.—(1957) *J. nat. Cancer Inst.*, **18**, 693.
VAN ZYL, A.—(1957) *J. Endocrin.*, **16**, 213.
WEISBURGER, J. H., WEISBURGER, E. K. AND MORRIS, H. P.—(1957) *Science*, **125**, 503.

hydroxyamines, and (2) the rate of certain hydroxylations in the liver may be increased during the stage of induction of cancer.

SUMMARY

Details are described for a method of estimating the trihydroxy-acids (THA) and dihydroxyacids (DHA) in rat bile, using reversed phase partition chromatography. The method was applied to the analysis of samples of rat bile, collected on the second to fourth days after cannulation of the bile duct from 39 control rats and 34 rats fed butter yellow (BY) for 63–153 days.

After cannulation the BY rats died sooner and lost weight faster than the controls. The cannulation and subsequent bile drainage gave rise in many rats of both series to bile duct hyperplasia and necrosis of the liver. The BY rats were very vulnerable to the effects of bile drainage and usually developed extensive liver damage about seven days after cannulation.

Although the secretion of THA was similar in both groups of rats, the BY rats showed a decrease in DHA for those rats with a daily secretion of THA exceeding 10 mg./100 g. body weight. The BY rats also showed an increase in bile volume, and a strong correlation between DHA and bile volume which was entirely absent in the controls. The proportion of DHA to THA rose as THA secretion was increased, especially in the control series. In the control series the nine rats with bile duct hyperplasia showed changes in bile composition characteristic of those induced by BY. Exclusion of the results from these rats greatly accentuated the difference in DHA between control and BY rats. In the BY series cannulation was followed by death quickest in those rats with the most abnormal bile composition.

The significance of the depression in DHA by BY feeding is discussed in terms of other conditions which affect bile acid ratios. A mechanism involving alterations in the rate of 12-hydroxylation is proposed to explain the effect of BY feeding on DHA, the increase in the proportion of DHA with a rise in THA, and the correlation in the BY rats between DHA and bile volume. The possible significance is examined of hydroxylations in general for the understanding of cancer.

Thanks are expressed to N. G. N. Matthews and J. Makunga for excellent technical assistance and to Miss J. van Veen for carrying out the cannulations. Mr. W. Lutz very kindly carried out the statistical analysis of the data. The study was greatly facilitated by a grant made to one of us (S. M.) by the National Cancer Association of South Africa, to which body grateful acknowledgment is made.

REFERENCES

Allen, M. J., Boyland, E., Dukes, C. E., Horning, E. S. and Watson, J. G.—(1957) *Brit. J. Cancer*, **11**, 212.

Bergström, S.—(1959) 'The Biosynthesis of Terpenes and Sterols'. A Ciba Foundation Symposium. London (J. A. Churchill & Co.) p. 185.

Idem and Danielsson, H.—(1958) *Acta physiol. scand.*, **43**, 1.

Idem and Sjövall, J.—(1951) *Acta chem. scand.*, **5**, 1267.—(1954) *Ibid.*, **8**, 611.

Berman, C.—(1958) *Advanc. Cancer Res.*, **5**, 56.

Booth, J. and Boyland, E.—(1957) *Biochem. J.*, **66**, 73.

Boyland, E.—(1958) *Brit. med. Bull.*, **14**, 153.

CAMERON, G. R. AND OAKLEY, C. L.—(1932) *J. Path. Bact.*, **35**, 769.
CAREY, J. B.—(1958) *J. clin. Invest.*, **37**, 1494.
CLAYSON, D. B.—(1953) *Brit. J. Cancer*, **7**, 460.
CONNEY, A. H., MILLER, E. C. AND MILLER, J. A.—(1957) *J. biol. Chem.*, **228**, 753.
COOK, J. W., KENNAWAY, E. L. AND KENNAWAY, N. M.—(1940) *Nature, Lond.*, **145**, 627.
DUNNING, W. F., CURTIS, M. R. AND MAUN, M. E.—(1950) *Cancer Res.*, **10**, 454.
ERIKSSON, S.—(1957) *Proc. Soc. exp. Biol. N.Y.*, **94**, 578, 582.
FIESER, L. F., GREENE, T. W., BISCHOFF, F., LOPEZ, G. AND RUPP, J. J.—(1955) *J. Amer. chem. Soc.*, **77**, 3928.
GILBERT, C., GILLMAN, J., LOUSTALOT, P. AND LUTZ, W.—(1958) *Brit. J. Cancer*, **12**, 565.
GILLMAN, J., GILBERT, C. AND SPENCE, L.—(1954) *Cancer*, **7**, 1109.
GREENSTEIN, J. P.—(1954) ' The Biochemistry of Cancer '. New York (Academic Press).
HAROLD, F. M., FELTZ, J. M. AND CHAIKOFF, I. L.—(1955) *Amer. J. Physiol.*, **183**, 459.
HARRIS, P. N. AND CLOWES, G. H. A.—(1952) *Cancer Res.*, **12**, 471.
HSIA, S. L., MATSCHINER, J. T., MAHOWALD, T. A., ELLIOTT, W. A., DOISY, E. A., JR., THAYER, S. A. AND DOISY, E. A.—(1957) *J. biol. Chem.*, **225**, 811 ; **226**, 667.—(1958) *Ibid.*, **230**, 573.
LINSTEDT, S. AND SAMUELSSON, B.—(1959) *Ibid.*, **234**, 2026.
MIRVISH, S.—(1957) *S.Afr. J. med. Sci.*, **22**, 158.—(1958) *Ibid.*, **23**, 33.
MOSBACH, E. S., KALINSKY, H. J., HALPERN, E. AND KENDALL, F. E.—(1954) *Arch. Biochem. Biophys.*, **51**, 402.
OSBORN, E. C., WOOTTON, I. D. P., DA SILVA, L. C. AND SHERLOCK, S.—(1959) *Lancet*, ii, 1049.
PASCHKIS, K. E., CANTAROW, A. AND STASNEY, J.—(1948) *Cancer Res.*, **8**, 257.
RUDMAN, D. AND KENDALL, F. E.—(1957) *J. clin. Invest.*, **36**, 530.
SIPERSTEIN, M. D., JAYKO, M. E., CHAIKOFF, I. L. AND DAUBEN, W. G.—(1952) *Proc. Soc. exp. Biol. N.Y.*, **81**, 720.
SJÖVALL, J.—(1953) *Acta physiol. scand.*, **29**, 232.
SPAIN, J. D. AND GRIFFIN, A. C.—(1957) *J. nat. Cancer Inst.*, **18**, 693.
VAN ZYL, A.—(1957) *J. Endocrin.*, **16**, 213.
WEISBURGER, J. H., WEISBURGER, E. K. AND MORRIS, H. P.—(1957) *Science*, **125**, 503.

SOME NEW DATA CONCERNING THE BIOLOGY OF TUMOURS.
The Effects of Heparin and its Components on Tumour Growth

G. CSABA, CECILIA HORVÁTH and Th. ÁCS

From the Institute of Histology and Embryology of the Budapest Medical University, Hungary

Received for publication February 11, 1960

The study of the mechanism of the agar-binding reaction—a new procedure for the diagnosis of cancer—has raised certain problems and it is hoped that their elucidation might throw fresh light upon the role of polysaccharides in the biology of neoplastic growth (Csaba and Törö, 1958 ; Csaba, Törö and Kiss, 1959*a*, 1959*b*). Heparin, contained in the serum, has been found to be an important factor in making the reaction positive or negative. Immune substances, produced by the organism in the proliferative stage of tumours, are in our opinion responsible for the positivity of the reaction, while heparin—by gaining ascendancy over the lipoproteins in the terminal phase of tumour-bearing subjects—causes the reaction to become neagative (Fig. 1). By way of hypothesis, it was suggested in our publication concerning the details of the mechanism of the agar-binding reaction (Csaba *et al.*, 1959) that the positivity of the reaction in the incipient phase of neoplastic growths was due to the predominance of serum lipoproteins arising from the tumours, or—in other words—that the demonstration of lipoproteins and the consequent positivity of the reaction were due to the fact that the amount of heparin was reduced. By artificially inhibiting the action of heparin in *in vitro* experiments we succeeded in turning the negative reaction given by the serum of healthy individuals into a positive one. Therefore we felt justified in instituting investigations into the role of heparinoid substances, particularly as the problem of the correlation between lipoproteins and tumours seems to have been sufficiently elucidated (Barclay *et al.*, 1957 ; Greenstein, 1954 ; Heiger, 1957 ; Holsti, 1958 ; Homburger and Fishmann, 1953 ; Rapport, *et al.*, 1958 ; Waterman, 1953). A few reports are available in respect of the role of heparinoid substances. It was, for instance, observed by Panizzari and Vegeto (1958) that the activity of heparin in the serum diminished after the implantation of the Walker tumour. Several reports contain data concerning high glucuronidase activity observable in tumours (Greenstein, 1945 ; Hamer, 1953). It is claimed by Kizer and McKoy (1959) that glucosamine, one of the most important structural components of heparin, is synthesized by homogenates of the Walker tumour from hexose 6-phosphate and glutamine.

Our present experiments were based on the notion that the amount of heparinoid substances contained in the serum becomes less during the proliferation of tumour cells, presumably because these substances are used up by the growing tumour. Accordingly, the experiments were started to cover two lines of approach: 1. We wanted to ascertain the effect of heparin and its components on tumour growth and the survival of inoculated animals ; 2. *in vitro* and *in vivo* experiments

to determine the effect of heparin-binding substances on the growth and viability of tumours.

The present paper deals with the effects produced by heparin and its components.

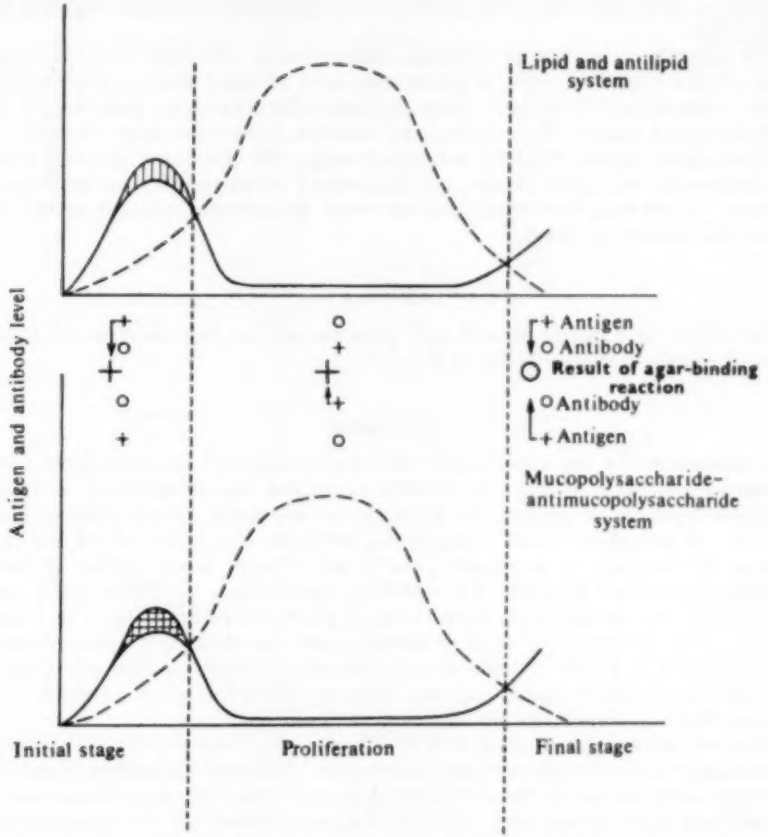


FIG. 1.—Correlation between the respective systems of lipids-antilipids and mucopolysaccharides-antimucopolysaccharides, and its effect on the result of the agar-binding reaction. The amount of lipids increases (shade lines) or that of the heparinoids decreases (squares) in the incipient phase of tumours.

METHOD

We studied the effect of heparinoid components on 220 tumour-bearing mice. Their average body weight was 20 g. The test animals—white and sand-coloured males and females—were taken from the stock of the National Institute of Public Health, Budapest. Transplanting Ehrlich ascites tumour into the mice, we used doses of 0·05 ml. for subcutaneous injections, i.e. approximately 60,000 cells

per mm.^{3}; the dose for intraperitoneal injections was likewise 0·05 ml. but the ascitic fluid was first diluted at the ratio 1 : 10, so that the number of cells was approximately 6000 per mm.^{3} in these cases. These doses were occasionally modified and are indicated in the tables. Low doses had the purpose of prolonging the tests so that the differences between experimentals and controls were more conspicuous.

The animals were treated with the subcutaneous administration of Heparin pulvis (Richter-80 I.U./mg.), *d*-glucuronic acid (Fluka) and α, *d*-glucosamine (Light) preparations. The daily doses of these substances were dissolved in 1 ml. of physiological saline. Tumour-bearing controls received a daily dose of 1 ml. of physiological saline, injected subcutaneously. We also used controls treated with glucuronic acid, glucosamine and heparinoid substances but without tumour injection. Other than the heparinoids to which the animals succumbed, the other substances showed no effect.

RESULTS

The effect of glucuronic acid and glucosamine on the survival of tumour-bearing animals is shown in Table I.

DISCUSSION

It was shown by the experiments that glucuronic acid, in daily doses varying between 5 mg. and 30 mg., invariably promoted the destruction of tumour-bearing animals in all groups, but failed to do any harm to non-tumour bearing controls. It therefore seems justified to attribute the observed effects to the presence of tumours as neoplastic growth was always more marked in treated than in non-treated animals. The effect of glucosamine appeared to be similar to, although less conspicuous than, that of glucuronic acid. That the observed effect must be principally due to glucuronic acid was strikingly demonstrated by the experiment in which both glucuronic acid and glucosamine were administered : their combined effect was in no way different from the effect observed in the animals which had received glucuronic acid alone.

That we were right in using only small amounts of ascites for the transfers is convincingly shown by the experimental results. Although high doses of glucuronic acid were administered to the members of group 7, the difference between experimentals and controls was very slight in this group where the transplanted ascites had not been diluted. Survival of the controls was considerably longer in group 6 while the survival of the experimentals was 25 per cent shorter than in group 7. Differences are most pronounced in the intraperitoneal groups 5 and 8 where the time of survival of the controls is very long and even reaches that of subcutaneously inoculated animals.

It was found by Heilbrunn (1956), Balázs and Holmgren (1947), and also by Koenig (1955) that whole heparin inhibits tumour growth. We failed to observe this effect because the doses of heparin applied in our experiments induced large haematomas and led to a quick death of the animals. At any rate, even if we accept the statement of these authors we must emphasize that their claim cannot apply to the structural components of heparin which, far from inhibiting tumours, promote neoplastic growth and so hasten the death of the animals. The experi-

TABLE I.—*Effect of Glucuronic Acid and Glucosamine on the Survival of Tumour-bearing Mice*

Group	Number of animals	Colour, sex	Route of administration	Treatment doses/mouse	Average survival days	Shortening of survival (Per cent)	Observations
1	20	White, F.	S.C.	Heparin 10 mg./day	—	—	5th–8th day exit.
	10	„ „	„	Control	35·0	—	—
2	10	White, F.	S.C.	Control	36·8	—	—
	10	Sand, M.	„	„	33·3	—	—
	10	„ „	„	„	32·9	—	—
	20	„ „	„	Gluc. ac. 10 mg./day	26·3	20·0	—
	20	„ „	„	Ditto	27·1	18·6	—
3	10	Sand, F.	S.C.	Control	33·3	—	—
	10	„ „	„	Gluc. ac. 10 mg./day	29·6	11·5	—
4	10	Sand, F.	S.C.	Control	32·9	—	—
	10	„ „	„	Gluc. ac. 10 mg./day	27·0	17·8	—
	10	„ „	„	Ditto	29·8	9·6	—
5	10	White, F.	I.P.	Control	33·4	—	—
	10	„ „	„	Gluc. ac. 5 mg./day	23·2	30·6	—
6	5	White, F.	I.P.	Control	20·0	—	Inoculated with undiluted ascites.
	5	„ „	„	Gluc. ac. 30 mg./day	15·0	25·0	—
7	10	White, F.	I.P.	Control	13·3	—	Inoculated with undiluted ascites.
	10	„ „	„	Gluc. ac. 30 mg./day	11·0	17·3	—
8	10	White, F.	I.P.	Control	33·4	—	Identical with control of group 5.
	10	„ „	„	Glucos. 2 mg. + gluc. ac. 5 mg./day	23·8	28·8	
9	10	White, F.	—	Gluc. ac. 30 mg./day for 30 days	—	—	Received no tumour; no effect observed.

mental results raise the problem : is the higher level of serum mucoids in the blood of tumour-bearing individuals and test animals with well-advanced tumours to be regarded as a consequence of neoplastic growth (Almquist and Lansing, 1957 ; Homburger and Fishmann, 1953 ; Rottimer, Levy and Conte, 1958 ; Shetlar *et al.*, 1949 ; Weimer *et al.*, 1957 ; Winzler and Smyth, 1948)—from the disintegration of mast cells etc.—or as the causative agent of tumours? There seems to be no sharp boundary between cause and effect : both seem to participate in the maintenance of the vicious circle in malignant disease.

It was not possible to resolve these problems on the evidence of the present experiments. Therefore, additional experiments—*in vitro* and *in vivo*—have been performed and the results form the subject of a subsequent publication.

SUMMARY

The effect of heparin and its components on the survival of tumour-bearing animals has been examined. The experiments, as described in the paper, show that glucuronic acid or glucosamine and glucuronic acid—if administered in protracted doses—shorten the life of tumour-bearing mice.

REFERENCES

ALMQUIST, P. O., AND LANSING, E.—(1957) *Scand. J. clin. Lab. Invest.*, **9**, 179.
BARCLAY, M., KAUFMAN, R. J., SVED, D. W., KIDDER, E. D., ESCHER, G. C. AND PETERMANN, M. L.—(1957) *Cancer*, **10**, 1076.
BALÁZS A., AND HOLMGREN, H.—(1947) *Proc. Soc. exp. Biol. N.Y.*, **72**, 141.
CSABA, G. AND TÖRÖ, I.—(1958) *Z. Krebsforsch*, **62**, 481.
Iidem AND KISS, F. I.—(1959) *Orientacion Medica*, **8**, 1.—(1959) *Orv. Hétil.*, **100**, 1580.
Iidem, JÓCSAI, G. AND ELÖDI, B.—(1959) *Neoplasma*, **6**, 366.
GREENSTEIN, J. P.—(1954) Biochemistry of Cancer '. New York (Academic Press).
HAMER, D.—(1953) *Brit. J. Cancer*, **12**, 661.
HEIGER, I.—(1957) *Proc. Roy. Soc.*, **147**, 84.
HEILBRUNN, L. V.—(1956) The Dynamics of Living Protoplasma '. New York (Academic Press).
HOLSTI, P.—(1958) *Naturwissenschaften*, **45**, 394.
HOMBURGER, F. AND FISHMANN, W. H.—(1953) ' The Physiopathology of Cancer '. New York (Holter).
KIZER, D. E. AND MCCOY, T. A.—(1959) *Cancer Res.*, **19**, 309.
KOENIG, H.—(1955) *Z. exp. Med.*, **126**, 67.
PANIZZARI, G. D. AND VEGETO, A.—(1958) *Arch. ital. Pat. clin. Tumori*, **2**, 1219.
RAPPORT, M., GRAF, L., SKIPSKI, V. P. AND ALONZO, N. F.—(1958) *Nature*, **181**, 1803.
ROTTIMER, A., LEVY, A. L. AND CONTE, A.—(1958) *Cancer*, **11**, 351.
SHETLAR, M. R., FOSTER, J. V., KELLY, K. H., SHETLAR, C. L., BRYAN, R. S. AND EVERETT, M. R.—(1949) *Cancer Res.*, **9**, 515.
WATERMAN, N.—(1953) *Bull. Ass. franç Cancer*, **40**, 340.
WEIMER, H. E., QUINN, F. A., REDLICH-MOSHIN, J. AND NISHIHARA, H.—(1957) *J. nat. Cancer Inst.*, **19**, 409.
WINZLER, R. J. AND SMYTH, J. M.—(1948) *J. clin. Invest.*, **27**, 617.

SOME NEW DATA CONCERNING THE BIOLOGY OF TUMOURS

The Effects of Heparin Inhibitors on Tumour Growth

G. CSABA, TH. ÁCS, CECILIA HORVÁTH AND ESZTER KAPA

From the Institute of Histology and Embryology of the Budapest Medical University, Hungary

Received for publication February 11, 1960

We have reported in our preceding communication (Csaba, Horváth and Ács, 1960) that the components of heparin shorten the life of tumour-bearing animals by promoting neoplastic growth. Since, according to the literature, these substances may be synthesized by the tumours themselves (Kizer and McCoy, 1959) and since—on the other hand—they accumulate in the serum of individuals with advanced tumours (Almquist and Lansing, 1957; Rottimer, Levy and Conte, 1958; Shetlar *et al.*, 1949; Weimer *et al.*, 1957; Winzler and Smyth, 1948), we thought it justified to ascertain whether and if so how the neutralization of these substances would affect the vitality of tumours. In accordance with our programme, described in the preceding communication, we neutralized the heparinoid substances in two kinds of experiments: in tissue cultures and in the living organism itself.

METHOD

In vitro experiments to test heparin inhibitors were performed in 600 tissue cultures, half of which were Maximow's double cover slip cultures and the other half flat-tube cultures (Törö, 1959). Fowl plasma coagulated with chick embryo extract, to which no heparinoid had been added, served as protective medium. A mixture of horse serum, chick embryo extract and Tyrode solution (3:1:6) was employed as culture medium for the controls. Toluidine blue was used as heparin inhibitor in the experimental cultures, this being added to the liquid medium to give a concentration of 1 γ/ml. or 10 γ/ml. (called "final concentration" in the following). It was either immediately or 48 hours after explantation that the washing fluid containing toluidine blue was added to the cultures. We observed the cultures during 10 days or fixed them within this period. The test cultures were obtained from C_3H, Guérin, Yoshida and Ehrlich tumours, while the control cultures were derived from the thymus, liver, spleen, lymph node, kidney, adrenal, brain, lung and heart of 16–17-day-old rat embryos and also of 3-day-old rats.

In vivo experiments to test heparin inhibitors were performed on 186 mice and 72 rats. The former were taken from the registered white and sand-coloured stock of the National Institute of Public Health, the latter from our own stock.

Guérin, Yoshida, Ehrlich ascites and Crocker S. 180 tumours were transplanted. As inhibitory substances, protamine-sulphate injection 1 per cent (Roche), toluidine blue (Gurr and Schuchard), thionine (Grübler), mixtures of the above

and, ammonium chloride (United Works of Pharmaceutical and Dietetic Products, Budapest) were applied.

At the outset, toluidine blue alone was used for the purposes of inhibition. It was first added to the food of the animals in a pulverized form. It was not well tolerated and the dose applied could not be estimated accurately. Therefore, we tried to administer the dye by way of a gastric tube. The technique of administration was rather unsatisfactory and quite a number of the animals died during the process of intubation which explains why there are certain groups in the tables with such a low number of test animals.

Having been convinced by the results of our preliminary experiments that, neither toluidine blue nor protamine sulphate alone were adequately efficient if given in tolerated doses, we had to start combined treatments. The tested combinations are denoted by the character " T ", i.e. the first letter of toluidine blue, their chief ingredient. The combined preparations bear the numbers T_1 to T_{11}, of which only T_5, T_4 and—to a lesser extent—T_{11} proved to be useful tumour inhibitors.

The preparation T_5 contains 10 mg./ml. of toluidine blue and protamine sulphate. Rats were given 10 mg./100 g. body weight of toluidine blue and 0·5 ml. per animal of protamine sulphate, the dye being dissolved in 1 ml. of distilled water. Mice received the same concentration of toluidine blue in the following doses : 0·8 ml. on the first, 0·6 ml. on the second, 0·4 ml. from the third day, and—in addition—0·1 ml. per animal of protamine sulphate.

The combined preparation T_4 contained 10 mg. of toluidine blue, 10 mg. of thionine and 10 mg. of ammonium chloride per ml. of distilled water. The reason for choosing these substances will be explained in the discussion. Rats were given 1 ml. per animal per 100 g. body weight, while mice received 0·4 ml. per animal per 20 g. body weight of this preparation. This combination contained no protamine sulphate.

The preparation T_{11} proved to be highly toxic and therefore not suitable for our experiments. It contained 20 mg. of toluidine blue, 10 mg. of thionine and 20 mg. of ammonium chloride per ml. of distilled water. As mentioned above, we also tested a few other combinations. These either produced no effect or were exceedingly toxic. The parenteral introduction of dyes, resulted in unsuccessful experiments.

RESULTS

Toluidine blue, applied in a final concentration of 10 γ/ml. in the *in vitro* experiments, either immediately inhibited the growth of the examined tumours, or did so in a very short time. The proliferation of tumour cells in the control cultures seemed to be especially intensive and conspicuous in the case of C_3H and Ehrlich tumours. Where the fluid containing toluidine blue was added to the cultures immediately after the explantation, only a few sporadic migrating cells appeared and these contained the dye in the form of coarse granules. In cases where the dye was applied 48 hours after the explantation, there was no difference between the growth of the test and control cultures until after the addition of the dye. Then isolated granules in the cells of the test cultures filled with the dye and *showed metachromasia* (purple). It should be noted that the growth of the cultures stopped forthwith so that—as regards growth—the picture seen 24 hours after the treatment did not differ from that seen at the beginning thereof.

A day after the application of the dye, it was still very finely distributed in some cells, while in others it appeared as coarse particles as were observed in cultures that had been treated immediately after explantation. No further growth could be observed even 48 hours after the treatment, while the controls continued to grow vigorously. It was at this time that the cells began to disintegrate in the treated cultures, a process which was completed 96 hours after the single treatment.

The binding of the dye in the cells is irreversible. If the culture is normally fed 24 hours after the first (and only) treatment and if also the subsequent washings are similar, proliferation will never occur again.

Toluidine blue in a final concentration of 1 γ/ml. gave less obvious results than the higher concentration. Only 1 or 2 granules were seen in the cells 24 hours after the treatment, and the tumour continued to grow, although less intensively than in the control cultures. The accumulation of granules became more pronounced 72 hours after the treatment; cellular disintegration began 96 hours after the first and 48 hours after the second treatment, to become complete by the end of the period of observation.

With one exception, none of the organs enumerated in the paragraph on method took up toluidine blue. This exception was the thymus. Like tumour cells, the cells of this organ—the epithelial membrane and the thymocytes—took up toluidine blue and disintegrated subsequently.

It should be noted that inhibition of growth by the dye was always more pronounced in the case of carcinomas than in that of sarcomas.

Results of heparin inhibition in *in vivo* experiments are tabulated (Tables I to XII). The first experiments in connection with Guérin's tumour were performed with large doses added to the food. Seeing that this method, while being efficacious, did not allow of the administration of precise doses we began administration by gastric tube. We took care, in our further experiments, to begin

TABLE I.—*Rats Inoculated with Guérin Tumour Subcutaneously*

Treatment begun 8 days after inoculation

Sex	Number of animals	Treatment	Duration (days)	Average weight tumour (g.)	Standard deviation (g.)	Average metastasis	Observation
F.	3	Control	—	26·13	±2·4	3·3	—
F.	5	Tol. blue 50 mg./day in food	13	12·0	1·5	0·62	Regionally only.

TABLE II.—*Rats Inoculated with Guérin Tumour Subcutaneously*

Treatment begun 48 hours after inoculation

Sex	Number of animals	Treatment	Duration (days)	Average weight tumour (g.)	Standard deviation (g.)	Average metastasis	Observation
F.	5	Control	—	4·44	1·0	187 mg.	—
F.	2	Tol. blue 10 mg./day	23	8·1 !!	1·42	250 „	Toxic phenomenon.
F.	2	T_4	23	0·085	0·012	—	„ „
F.	3	T_5	23	0·115	0·018	—	„ „

TABLE III.—*Rats Inoculated with Guérin Tumour Subcutaneously*

Treatment begun 13 days after inoculation

Sex	Number of animals	Treatment	Duration (days)	Average weight tumour (g.)	Standard deviation (g.)	Inhibition (%)	Observation
M.	4	Control	—	8·72	1·68	—	—
M.	6	T_5	7	6·64	1·2	24·2	—

TABLE IV.—*Rats Inoculated with Guérin Tumour Subcutaneously*

Treatment begun 19 days after inoculation

Sex	Number of animals	Treatment	Duration (days)	Average weight tumour (g.)	Standard deviation (g.)	Average metastasis	Observation
M.	2	Control	—	18·15	3·2	9·25	Abdominal cavity filled with metastasis.
M.	2	T_5	7	5·85	1·8	2·65	Metastasis regionally only.

TABLE V.—*Rats Inoculated with Guérin Tumour Subcutaneously*

Treatment begun 28 days after inoculation

Sex	Number of animals	Treatment	Duration (days)	Average weight tumour (g.)	Standard deviation (g.)	Average metastasis	Inhibition (%)
F.	3	Control	—	14·1	1·63	1·5	62·4
F.	5	T_5	7	5·3	0·74	3·0	—

TABLE VI.—*Mice Inoculated with 0·05 ml. of Ehrlich Ascites Tumour Subcutaneously*

Treatment begun 24 hours after inoculation

Sex	Number of animals	Treatment	Duration (days)	Average weight tumour (g.)	Observations
F.	10	Control	—	3·8	—
M.	13	,,	—	1·89	—
F.	10	Tol. blue 25 mg./day +prot. sulph. 0·1 ml./day	17	1·88	Dose toxic; only 7 animals alive at examination.
M.	20	Ditto	17	1·46	Dose strongly toxic; only 3 animals alive at examination.
M.	20	Tol. blue 25 mg./day	17	1·83	Dose strongly toxic; only 3 animals alive at examination.

TABLE VII.—*Mice Inoculated with 0·1 ml. of Ehrlich Ascites Tumour Subcutaneously*

Treatment begun 9 days after inoculation

Sex	Number of animals	Treatment	Duration (days)	Average weight tumour (g.)	Standard deviation (g.)	Inhibition (%)	Observation
M.	8	Control	—	3·41	0·63	—	—
M.	10	T_{11}	14	1·87	0·41	45·5	—

TABLE VIII.—*Mice Inoculated with 0·2 ml. of Ehrlich Ascites Tumour Subcutaneously*

Treatment begun 12 days after inoculation

Sex	Number of animals	Treatment	Duration (days)	Average weight tumour (g.)	Standard deviation (g.)	Inhibition (%)	Observation
M.	4	Control	—	1·7	0·37	—	—
M.	31	T_4	7	0·91	0·14	46·5	—

TABLE IX.—*Mice Inoculated with 0·1 ml. of Ehrlich Ascites Tumour Intraperitoneally*

Treatment begun 24 hours after inoculation

Sex	Number of animals	Treatment	Duration (days)	Amount of ascites (ml.)	Cell count in 1 ml. of ascites	Average cell count per mouse	Observation
M.	10	Control	—	3·73	99,980	$373,10^4$	—
M.	10	Tol. blue 0·4 mg. +prot. 0·1 ml./day	8	2·78	92,800	$258,10^4$	Three mice died.
M.	10	T_4 0·8 ml., then 0·4 ml.	5 8	1·5	114,600	—	Six mice died during treatment; no ascites in 2 mice.

TABLE X.—*Mice Inoculated with 0·1 ml. of Ehrlich Ascites Tumour Subcutaneously*

Treatment begun simultaneously with inoculation

Sex	Number of animals	Treatment	Duration (days)	Average weight of tumour (g.)	Standard deviation (g.)	Inhibition (%)	Observation
M.	4	Control	—	1·0	0·25	—	—
M.	3	T_8 bound to polyvynil pyrolidone	10	0·43	0·05	57	Two out of 5 animals died.

TABLE XI.—*Mice Inoculated with Crocker S. 180 Subcutaneously*

Treatment begun 10 days after inoculation

Sex	Number of animals	Treatment	Duration (days)	Average weight of tumour (g.)	Standard deviation (g.)	Inhibition (%)	Observation
M.	13	Control	—	3·0	0·7	—	—
M.	10	T_{11}	7	1·52	0·52	49·5	Three mice died during process of feeding.

TABLE XII.—*Rats Inoculated with Yoshida Tumour Subcutaneously*

Treatment begun 4 days after inoculation

Sex	Number of animals	Treatment	Duration (days)	Average weight of tumour (g.)	Standard deviation (g.)	Inhibition (%)	Observation
M. and F.	10	Control	—	9·6	2·7	—	—
Ditto	10	T_4	7	5·5	1·35	42·7	Double dose on 1st day.
„	10	T_3	7	5·04	1·58	47·0	Double dose of tol. blue on 1st day.

treatment at different times, i.e. to obtain tumours with different degrees of vitality. Figures of measurements, percentage of inhibition (wherever such calculations were deemed to be justified by the number of animals) are indicated in the tables ; special observations are contained in a separate column.

Ehrlich ascites tumours were transplanted partly through the subcutaneous and partly through the intraperitoneal route. As has been mentioned, the purpose of low dosage was a longer survival of the animals.

Only a few groups were inoculated with Crocker S. 180 and Yoshida tumours because results obtained in these cases were in perfect agreement with those observed in connection with the other two kinds of tumours and also because the number of animals in the particular groups seemed to suffice for adequate conclusions.

DISCUSSION

It was suggested in our previous publication that heparinoids may promote neoplastic growth or that they may even be regarded as causative agents. Our present experiments with heparin inhibitors throw a much sharper light upon the role played by polysaccharides.

It was proved by these experiments that even extremely low concentrations (10^{-5} g) of toluidine blue are capable of inhibiting tumour growth and destroying tumour cells in tissue cultures. Apart from this, it was shown—a fact of great theoretical importance—that what happens in the cells is not a mere storage of toluidine blue, followed by removal by phyagocytes, but the binding of heparin, a substance which is essential for the proliferation of the tumour cells. That this is so is borne out by the fact that the dye shows metachromasia in the cells.

The balance of the experimental results allows the conclusion that heparin is synthesized by and utilized for the growth of malignant tumour cells. There are, therefore, three alternatives open for us if we want to stop tumour growth *in vivo* :

1. The binding of some cytotoxic substance to glucuronic acid or heparinoid : its selective accumulation in the tumour cells should lead to their destruction.

2. The suppression of the organism's heparinoid substances with the consequent inhibition of tumour growth.

3. A combination of 1 and 2, i.e. binding of the organism's own heparinoids, especially of the heparin which circulates in the blood, followed by the introduction

into the organism of heparinoids bound to a cytotoxic substance for which—on account of the bound condition of the organism's own heparinoids—the affinity of the tumour has increased.

Seeing that method 1 requires chemical operations that will be performed at a later date, we began the examination of method 2, little suspecting that, by doing so, we were to arrive at method 3, which then proved the most useful.

Results assembled in the tables make it clear that the binding of the heparinoid substances strongly inhibits tumour growth. The percentage of inhibition is usually about 45 per cent but reached in one case as much as 62·4 per cent (Table V).

The potency of the inhibitory effect seems to depend on the rate of growth of each particular tumour, the time between tumour inoculation and the beginning of treatment, and on the size of the tumour at this time. The nature of the tumour does not appear to have a decisive influence on the strength of the effect, for high values of inhibition were obtained with every kind of tumour. We concluded from our experiments that the best results could be expected if (1) treatment were started immediately after the implantation of the tumour ; (2) slowly-growing tumours were treated ; (3) the treatment were protracted.

We feel justified in claiming that our method, elaborated upon the basis of theoretical considerations, yields satisfactory results in the case of transplantable tumours. It seems nevertheless necessary to discuss a few problems which influence the success of the treatment.

It is, first of all, very important that adequate doses be used. We observed that the growth of the tumours did not begin to decrease immediately after the treatment : it required 6–7 days for the animals to become saturated with the dye. It was then that proliferation slowed or stopped and necrosis occurred.

Why is combined treatment more satisfactory than the other methods? Neither toluidine blue (in non-toxic doses) nor protamine sulphate alone produce inhibition, while their combined application never failed to give satisfaction. This raises the theoretical possibility that the essential point of the experiments was more than the simple binding of heparinoid substances, namely the combination of methods 1 and 2. Experiments performed on rabbits (unpublished) have shown that protamine sulphate is capable of strongly diminishing the heparin level of the blood for a period of 6–10 hours. The absorption of toluidine blue is slower ; this was administered perorally and not parenterally as the protamine sulphate. It is, hence, safe to assume that, during the time of absorption, toluidine blue does not—or only to a negligible extent—combine with the heparinoids of the blood so that the dye is much more bound in the tissues. This would mean that, after some time, the heparinoids of the tissues—including those around the tumours—become bound to toluidine blue. The binding is very strong. It is, on the other hand, conceivable that as long as the heparinoid substances of the blood are bound by protamine sulphate, the tumours are able to take up only those heparinoids which are bound by toluidine blue. Provided it is true that toludine blue accumulated in the cells produces a toxic effect—and the results of experiments with tissue cultures confirm this assumption—all that actually happens is that we are binding the organism's own heparinoids to a cytotoxic agent by means of a biological procedure so that, circulating heparinoids being bound, the affinity of tumours for those heparinoid substances which are bound by toluidine blue and situated in their immediate vicinity becomes more pronounced.

The question arises here: why is there such a great amount of toluidine-blue-bound heparinoids around the tumours? The answer is simple enough. The number of mast cells increases in the neighbourhood of tumours (Asboe-Hansen, 1954; Asboe-Hansen, Levi and Wegelius, 1957; Cramer and Simpson, 1944; Csaba, Törö and Kiss, 1959; Koenig, 1955; Lascane, 1958); these cells contain heparinoid substances which combine with toluidine blue. The affinity of the mast cells for heparin-binding substances is very strong, perhaps stronger even than that of tumour cells; hence, if we require that there should remain a sufficient amount of heparinoids for the tumour cells beyond what has been bound by the mast cells, the doses used in the treatment must be adequate, and—equally—the duration of the treatment itself must be long enough. Since the uptake of heparinoids by the mast cells weakens the tissue barrier which helps to prevent tumour growth, doses must be such as to damage not only the mast cells but the tumour cells as well.

It may be presumed that the reasons why the effect of dyes does not manifest itself until the 6th or 7th day following treatment is that this time is needed for the organism to acquire sufficient toluidine-bound heparinoid as to enable the tumour to utilize this material.

Another form of treatment tested by us was the combined administration of toluidine blue, thionine and ammonium chloride. We were led by the following considerations in doing so: (1) Thionine, too, combines with heparinoids, although not quite as specifically as toluidine blue. As it is less toxic it can be well employed together with toluidine blue without increasing the toxicity of the latter. (2) A histological observation was our second reason: we found that a reduction of the pH value during the process of staining with toluidine blue helped to make the staining of mast cells more specific. This induced us to use ammonium chloride for acidification *in vivo*, and the result proved to be satisfactory.

The objection might be raised that the tumour-reducing effect of heparin inhibitors is due to a general intoxication of the organism produced by these substances. We think that their specific action is well proved by the electivity observed in the course of experiments with tissue cultures, further by the fact that we succeeded in inhibiting tumours in living animals without any sign of a general intoxication; and—finally—by the fact that the administration of easily tolerated doses of the two substances (protamine sulphate and toluidine blue) proved to be more effective than either of them administered independently in toxic doses.

Our experiments have thus furnished evidence to show that tumours utilize or synthesize heparinoid substances required for their growth. Led by such

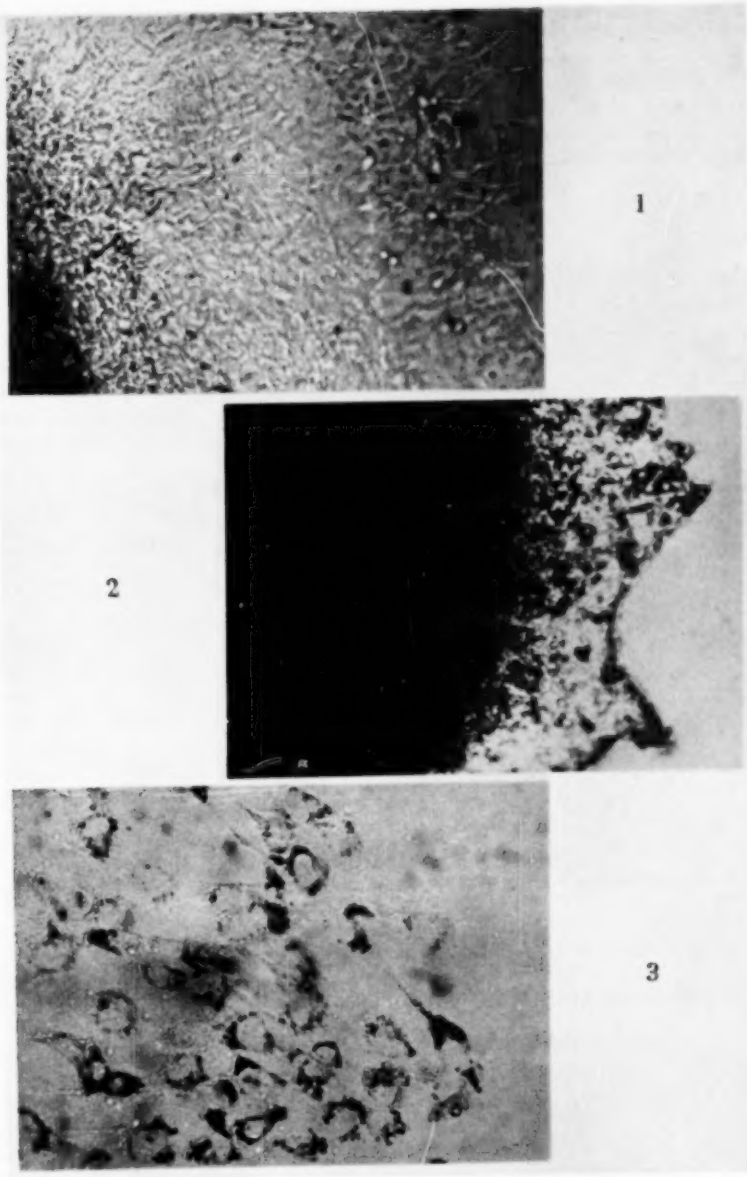
---

EXPLANATION OF PLATE

FIG. 1.—C_3H-culture 72 hours after explantation and 24 hours after normal feeding. Control. Unstained. ×75.

FIG. 2.—C_3H-culture placed in a liquid medium containing 10 γ/ml. of toluidine blue immediately after explantation and fed with the same fluid after 48 hours. The photgraph was taken 72 hours after explantation. Accumulation of metachromatic toluidine blue observable in cells. Unstained. ×75.

FIG. 3.—C_3H-culture, 72 hours after explantation and 24 hours after feeding with toluidine blue in a final concentration of 10 γ/ml. Many metachromatic granules of toluidine blue observable in cells. Unstained. ×75.



Csaba, Ács, Horváth and Kapa.

theoretical considerations we succeeded in elaborating a method for the inhibition of tumour growth which has proved successful in animal experiments. The substances employed by us are antimetabolites rather than cytostatic substances. That this is true is shown by the fact that cytostatic substances are known to affect quickly-dividing tumour cells in the first place, while our method produces a more marked effect if cell proliferation is slow. Far from being inconvenient, this feature of our method is decidedly advantageous if we remember that—apart from haemoblastoses—tumours in human subjects are frequently slower growing than transplantable animal tumours.

SUMMARY

Relying on the evidence of *in vitro* and *in vivo* experiments the authors conclude that tumours require heparinoid substances for their growth, and describe new possibilities for the inhibition of tumour growth. Theoretical considerations have led to the elaboration of a method by which it is possible to check the proliferation of transplantable tumour cells in about 45 per cent of the test cases. These theoretical considerations, substantiated by experimental results, open up a fresh path to new therapeutic experiments.

The authors received valuable assistance in their experiments from the staff of the United Works of Pharmaceutical Dietetic Products, Budapest, for which they want to express gratitude.

REFERENCES

Almquist, P. O. and Lansing, E.—(1957) *Scand. J. clin. Lab. Invest.*, **9**, 179.
Asboe-Hansen, G.—(1954) 'The mast cell' in 'International Review of Cytology', Ed. Bourne and Danieli, New York (Academic Press) Vol. 3).
Idem, Levi, H. and Wegelius, O.—(1957) *Cancer Res.*, **17**, 792.
Cramer, W. and Simpson, W. L.—(1944) *Ibid.*, **4**, 601.
Csaba, G., Horváth, C. and Ács, Th.—(1960) *Brit. J. Cancer*, **14**, 362.
Idem, Törö, I. and Kiss, F. I.—(1959) *Orientacion Medica*. **8**, 1.
Kizer, D. E. and McCoy, T. A.—(1959) *Cancer Res.*, **19**, 309.
Koenig, H.—(1955) *Z. exp. Med.*, **126**, 67.
Lascane, E. F.—(1958) *Cancer*, **11**, 1110.
Rottimer, A., Levy, A. L. and Conte, A.—(1958) *Ibid.*, **11**, 351.
Shetlar, M. R., Foster, J. V., Kelly, K. H., Shetlar, C. L., Bryan, R. S. and Everett, M. R.—(1949) *Cancer Res.*, **9**, 515.
Törö, I.—(1959) Szövettenyésztés (Tissue Cultures) In A. Kovách's book: 'Methods of Examination in Experimental Medicine'. Budapest (Hungarian Publ. House of the Academy).
Weimer, H., Quinn, F. A., Redlich-Moshin, J. and Nishihara, H.—(1957) *J. nat. Cancer Inst.*, **19**, 409.
Winzler, R. J. and Smyth, J. M.—(1948) *J. clin. Invest*, **27**, 617.

HYPERCITRICEMIA IN HUMAN CANCER FACTORS CONCERNED IN PATHOGENESIS AND TREATMENT

H. M. LEMON,* J. H. MUELLER,† J. M. LOONEY, W. H. CHASEN AND MARCIA KELMAN

From the Division of Neoplastic Disease, Department of Medicine, the Department of Biochemistry, Boston University School of Medicine and the Outpatient Clinic, Boston Veterans Administration, Massachusetts, U.S.A.

Received for publication January 12, 1960

PATIENTS with leukemia, metastatic carcinoma and sarcoma possess altered carbohydrate metabolism as shown by reduced glucose tolerance (Marks and Bishop, 1957), and elevated resting venous lactic acid (Cori and Cori, 1925). Enzymes concerned in tissue glycolysis are often increased in activity in serum during neoplastic disease progression, including phosphohexose isomerase (Bodansky, 1954*b*), aldolase (Sibley Fleischer and Higgins, 1955), and lactic dehydrogenase (Hill and Levi, 1954). Acid phosphomonoesterases hydrolyzing three-carbon substrates produced during glycolysis are also increased in activity in venous blood from patients with breast and prostate cancer (Reynolds, Lemon and Byrnes, 1956). Observations indicating possible abnormalities of the Krebs tricarboxylic acid cycle in human cancer patients however are limited to increased DPN-dependent dehydrogenase activity in sera of patients with hepatic metastases (Wolfson, Spencer, Sterkel, and Williams-Ashman, 1958; Schwartz, Greenberg, and Bodansky, 1959), and a single report of abnormal venous citric acid (Kyle and Canary, 1957). In this communication we are reporting hypercitricemia as a frequent abnormality of untreated advanced cancer. A preliminary account of these observations has been published elsewhere (Lemon, Mueller, Looney, Chasen and Kelman, 1959).

METHODS

Citrate concentration in blood obtained from various sites has been determined utilizing the Ettinger modification of the highly specific pentobromoacetone method (Ettinger, Goldbaum and Smith, 1952). Blood samples were obtained from volunteers and patients following a 12-hour fast and analyses performed in duplicate in most instances. Chloral hydrate which is the only known drug possibly reacting to this proceedure has not been administered within 12 hours to most of the patients or any of the volunteers studied. As a further check on the methods used, citric and other organic acids have been separated and concentrated from sera by a method developed by Dr. H. H. Wotiz, using 4N KOH to precipitate serum proteins followed by neutralization to pH 6·0 with concentrated HCl. The precipitate was centrifuged and washed once with distilled H_2O. The washings combined with the supernate were made slightly alkaline

* Present address: Massachusetts Memorial Hospitals, 750 Harrison Avenue, Boston 18, Massachusetts.

† Present address: National Naval Medical Center, Bethesda, Maryland.

with 2·5 N NaOH, and reacidified by bubbling CO_2 through the solution. These extracts were evaporated to dryness, resuspended in 1·0 c.c. ethanol and then chromatographed, along with reference citrate standards (Stark, Goodbar, and Owens, 1951). The citrate zone was then eluted, and the Ettinger procedure applied to the extract. A close agreement with the original analysis of unextracted serum was usually obtained in sera from cancerous and non-cancerous patients, indicating that citrate rather than some other organic acid was actually being measured.*

Blood was obtained in some volunteers and patients by simultaneous posterior iliac crest aspiration, arterial puncture and venipuncture. Following the initial sample 0·5 g. of sodium citrate as a 4 per cent solution was injected intravenously in a two minute period in many patients, with additional venous samples obtained at 5, 10, 20 and 30 minutes following commencement of injection. Since it was soon found that citrate concentration decreased rapidly with time between 5–30 minutes after injection, comparable to a first order reaction (Lemon, Mueller, Looney, Chasen and Kelman, 1959), only 5 and 30 minute samples were routinely collected for measurement of clearance rates. Analysis of serum calcium (Clark and Collip, 1925), phosphorus (Fiske and Subbarow, 1925) and glucose before and 30 minutes following injection was performed on a sample of 40 patients. Periodic serum calcium, phosphorus and alkaline phosphatase (Bodansky, 1932) analyses were performed in addition on most of the cancer patients. Simultaneous observations of copper-resistant serum acid phosphatase, (Reynolds, Lemon and Byrnes, 1956) and glutamic oxalacetic transaminase, (Franco, 1957) were made on the same serum sample, in a representative series comprising many of the cancer patients.

Patients were classified according to the nature of their principal disease. Biopsy proof of cancer was obtained in all the cancer patients investigated, and the extent and location of their metastases was assessed from clinical and radiologic diagnostic procedures. Table I summarizes the diagnostic information on the patients studied.

In the statistical evaluation of data, only the initial serum-observation was utilized for the comparison of means between different groups of patients with various diseases. This tends to underestimate the frequency of elevated blood citric acid, which is more frequent in the more advanced stages of cancer, but is necessary for a true comparison between cancer and other diseases, in which only a single observation was available for each case. Hypercitricemia has been defined as serum citrate value exceeding twice the standard deviation of the mean of healthy volunteers of the same sex, a value which is at the 95 per cent level of confidence for abnormality.

RESULTS

1. *Citrate dynamics*

The increase of serum citrate from 0–5 minutes ($\triangle$) was regarded as the result of rapid dilution of the 0·5 g. dose into plasma and extracellular fluid, an

* The analytic procedure has repeatedly yielded 95–98 per cent recovery of citrate added to whole blood or serum. Excellent checks within 2–3 per cent accuracy have been obtained when duplicate analyses for citrate were carried out using the method reported by Saffron and Dendstadt (1948). All sera were immediatley frozen upon separation from clot and analyses should be carried out within a few days of collection. Frozen sera may show 5–10 per cent changes in citrate concentration with prolonged storage. Simultaneous citrate standards were routinely run with each group of analyses.

TABLE I.—*Classification of Clinical Material*

Diagnosis	Source of material	Number of patients	Nutrition	Stage of Disease
Healthy volunteers .	Medical students. Hospital personnel	71	Excellent	—
Rheumatoid and degenerative arthritis	Ambulatory out-patients, with 15-year documented history	32	Good	Stage II. III. American Rheumatism Association.
Non-cancer disorders .	Chiefly hospital patients, some bed-fast	33	Good to fair	—
Pre-malignant lesions and benign tumours	Ambulatory out-patients and hospital in-patients	28	Excellent to good	—
Carcinoma and sarcoma	Ambulatory and bed-fast hospital patients	195	Good to fair	All stages, from early localized surgically cured to distant metastases.
Total . .	—	358	—	—

assumption which appears valid (Bunker, Stetson, Coe, Grillo, and Murphy, 1955). This distribution space S was determined by dividing the total in μg. ($0{\cdot}5 \times 10^6$) by $\triangle$ given in μg. per ml. In healthy volunteers and cancer patients the mean for this space was 21·6 liters for males and 14·0 liters for females (Table II).

Following the peak concentration of citrate 5 minutes after start of injection serum citrate concentration rapidly declined in a semilogarithmic manner compatible with a first order reaction, the initial baseline concentration very nearly being attained in normal, arthritic and cancer patients at 30 minutes time (Lemon, Mueller, Looney, Chasen and Kelman, 1959). The rate of decrease of serum citrate concentration approximated 1 μg./ml./min., which when multiplied by S $\times$ 60 in each case provided an estimate of the hourly rate of citrate clearance from plasma and extracellular fluid, by diffusion, metabolism, and renal excretion. In normal males, plasma clearance approximated 900 mg. per hour and in females, 800 mg. per hour. Urinary citrate excretion was not measured, since preliminary studies showed poor correlation between serum and urine citrate concentration. Citrate excretion in the urine appears to fluctuate quite independently of serum values owing to high renal uptake and metabolism (Herndon and Freeman, 1958). Only a small fraction of plasma citrate passing through the kidney is excreted, the amount being affected by acid-base balance and vitamin D content of the diet (Yarbo, 1956).

2. *Fasting venous citrate concentrations and disease*

In conformity with observations published by Rechenberger and Benndorf while this study was underway (Rechenberger and Benndorf, 1956), the mean venous citrate concentration of healthy females was found to exceed that of males of comparable age groups (Fig. 1 ; Tables II, III). The mean fasting venous concentration of male and female patients with rheumatoid and osteoarthritis was almost identical to that of their sex-matched volunteer controls.

Patients with benign tumours such as benign prostatic hyperplasia and mammary dysplasia had citrate concentrations also within the normal range for their

TABLE II.—*Observations of Citrate Dynamics in Healthy Volunteers and Patients with Benign Diseases*

Group	Sex	Number of cases	Mean fasting venous citrate concentration	Range	Frequency of observation above 2×S.D.	Distribution space (liters ±S.E.)	Clearance rate (mg./hr. ±S.E.)
Normal							
Volunteers	M.	27	27·2±1·3 ($p<0·001$)*	16·4–45·7	1	21·6±2·4 ($p=0·012$)*	896±58
	F.	44	38·3±1·8	..	2	14·9±1·3	799±38
		71			3=4·2%		
Non-cancer							
Hepatic cirrhosis	M.	3	33·9	18–61·1	1	..	..
	F.	4	46·5	34·8–51·0	0	..	..
Osteo-porosis	„	2	..	20·2–38·4	0	..	..
Pregnancy	„	3	25·0	14·4–31·0	0	..	..
Other disease	M.	10	34·5±5·7	14·8–84·9	1	..	..
	F.	10	39·1±4·8	20·4–64·4	1	..	..
Rheumatoid arthritis	M.	23	27·9±1·2	20–39	0	31·4±4·8	1027±33
Osteo- and degenerative arthritis	„	9	26·5±0·4	23·5–30·5	0	26·5±2·1	955±71
		65			3=4·6%		
Pre-malignant							
Mammary dysplasia	F.	13	30·4±2·7	16·1–49·9	0	..	..
Gynecomastia	M.	2	—	24·0–58·5	1	..	..
Benign prostatic hyperplasia	„	12	29·0±2·6	15·7–44·8	2	..	..
		27			3=11·1%		
Grand total	..	163	..	..	9=5·5%	..	..

* Significance between upper and lower figures.

sex. However, a few patients with carcinomas amenable to surgical excision and with clinically detectable metastases showed abnormal elevation of fasting venous citrate concentrations (Table III).

Women of all age groups with untreated metastatic carcinoma of the breast, had fasting venous citrate concentrations significantly ($p = < ·01$) in excess of volunteers, arthritic females or women with mammary dysplasia (Tables II, IV). Concentrations of venous citrate often exceeded more than 57 μg./ml. in untreated patients with advanced disease or patients in terminal relapse following a hormone-induced disease remission. Equally high citrate concentrations were noted in male patients with a variety of other types of metastatic carcinoma, including bronchogenic and pulmonary carcinoma, gastrointestinal carcinoma, fibrosarcoma, and lymphoma (Table III). Hypercitricemia of this degree has been noted only

rarely in non-cancer patients, including one obese female with mild uncontrolled diabetes mellitus, an anxious male with questionable peptic ulcer symptoms and negative X-ray findings, and one case of osteogenesis imperfecta.

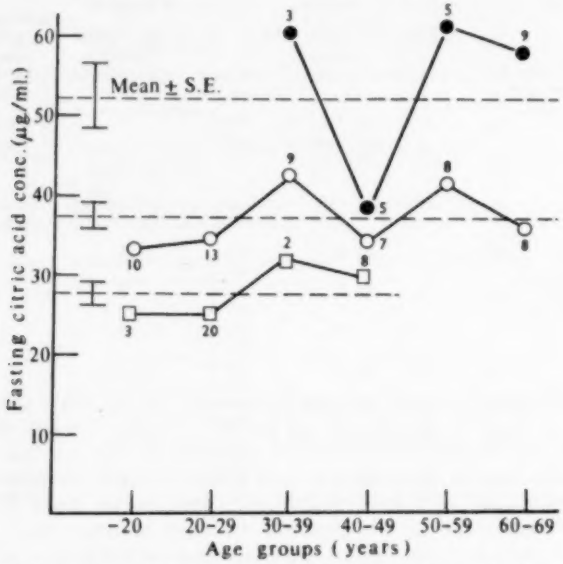


FIG. 1.—Mean fasting serum citric acid concentration in healthy volunteer controls and untreated metastatic breast cancer in females, grouped by age decade. Small numbers at each point indicate number of patients observed. Horizontal dashed line indicates mean, with range of standard error indicated by upright bracket at left, for the entire series of patients in each of the three classifications.

●——● Untreated metastatic breast cancer (24).

Controls

○——○ Female (55).

□——□ Male (37).

The discrepancy between the total cases and the sums at each point in this chart represents those rare cases for whom age was unknown from available records and solitary cases of other age decade categories which were not suitable for plotting as means.

3. *Citrate clearance rate and disease*

No significant sex difference was noted in mean citrate clearances (Table II). Male patients with extensive rheumatoid arthritis cleared injected citrate from their blood stream at rates which did not differ significantly from male or female normal volunteers. There was no significant deviation from volunteers in the average rate of citrate clearance by patients with various cancers other than breast, except for males with inactive post-therapy non-breast cancer, who showed a subnormal mean clearance rate in the small group tested (Table III). However, untreated patients with disseminated breast cancer tended to have the highest mean rate of citrate clearance, of any of the groups studied, as well as the highest

TABLE III.—*Observations of Citrate Dynamics in Patients with Cancer*

Group	Sex	Number of patients	Mean fasting venous citrate (μg/ml. ± S.E. initial observation)	Range	Frequency of observations exceeding 2 × S.D.	Number of patients	Distribution space (liters ± S.E.)	Clearance rate (mg./hr. ± S.E.)
Non-breast Cancer								
Local, pre-operative .	M.	5	36·3±5·6	15·7–50	3	..	..	..
	F.	1	..	72·5	1	..	..	..
Inactive post-therapy	M.	7	36·0±4·3	16·9–52·9	2	8	18·7±3·6	490±107
	F.	9	38·8±7·2	16·9–76·1	3	6	19·4±4·1	1013± 90
Active distant metastases	M.	42	42·0±3·2	15·5–100	14	12	15·8±2·1	828±127
	F.	35	35·7±2·2	12·1–69·9	3	8	18·2±2·6	715±130
Carcinoma of prostate	M.	8	37·6±5·9	14·9–66·5	3	..	..	..
		107			29=27%			
Carcinoma of Breast								
Local, pre-operative .	F.	6	27·8±2·7	20·7–39·5	0	..	..	..
Inactive, post-therapy	,,	20	36·5±1·7	22·3–51·4	0	..	..	..
Active distant metastases no therapy	,,	41	52·1±3·5	19·4–116·6	11	18	18·2±2·7	992 ±72
Active metastases treated by sex hormones	,,	3	..	22·9–72·6	1	..	..	($p<0·01$)*
Active metastases treated by cortisone or prednisone	,,	18	46·2±5·6	19·4–93·0	4	26	15·5±1·5	720± 59
		88			16=18·4%			
Grand total .		195			45=23%	78		

* Significance of difference between upper and lower figures.

mean fasting venous citrate. Following therapy with adrenal corticoid hormones, breast cancer patients showed a reduction of citrate clearance rates, as well as a reduction in fasting citrate level.

In one patient with breast cancer, citrate clearance was simultaneously measured in mixed venous blood, and in venous blood passing through the primary tumour. The citrate clearance rate was identical in both areas, according to our method of calculation (Table IV). This suggests that the difference in blood citrate concentration in the two sites was not due to uptake of citrate by the neoplasm.

These results would suggest increased citrate diffusion from some body tissue accounting for the increased blood level, rather than reduced tissue utilization in carcinomatosis. Bone which contains over 1 per cent citrate (dry weight), in its organic matrix (Dickens, 1941 ; Thunberg, 1953) is one of the more obvious sources for citrate release, but metastatic cancer and malignant tumour cells themselves contain considerable amounts of citrate (Potter and Busch, 1950 ; Dietrich and Shapiro, 1956 ; Miller and Carruthers, 1950).

4. *Citrate distribution space and disease*

A sex difference was noted in the mean distribution space of healthy volunteers, which was statistically significant at a level of confidence ($p = \pm .012$; Table II).

TABLE IV.—*Acid Phosphatase Activity and Citrate Concentration in Venous Blood Draining Breast Cancer Primary Site, Compared to Mixed Venous Blood*

Date	Time	Venous blood from left breast primary tumor		Mixed venous blood from right antecubital vein	
		Citrate (μg./ml.)	Acid phosphatase (μmole/100 ml.)	Citrate (μg./ml.)	Acid phosphatase (μmole/100 ml.)
1/13/58	Fasting	15	27·6	26·9	13·6
	5 minutes after citrate injection	23·5	39·1	70·0	30·2
	30 minutes after citrate injection	—	25·8	34·5	16·4
1/16/59	Fasting	16·5	29·4	33·0	25·6
	7 minutes after injection	—	—	65·5	18·8
	10 minutes after injection	33·5	27·2	—	—
	26 minutes after injection	—	—	35·5	24·5
	29 minutes after injection	19·5	28·3	—	—
Mean values		21·6	29·6	44·2	21·5
Citrate dynamics 1/13/58	Distribution space	—		11·6 liters	
	Clearance rate	—		990 mg./hr.	
1/16/58	Distribution space	29·4 liters		13·7 liters	
	Clearance rate	1300 mg./hr.		1300 mg./hr.	

A sex difference was not seen however in either of two groups of non-breast cancer patients, in whom the average male distribution space approached the female value. Male rheumatoid arthritic patients, on the other hand possessed a higher mean distribution space for citrate than healthy volunteers, and were significantly different in this respect from all groups of male or female patients with active metastatic cancer of breast as well as other types of cancer ($p = < \cdot 012$). These results suggest that the tissues of patients with rheumatoid arthritis are more widely and rapidly permeable to injected citrate resulting in a 5 minute post-injection peak value which is considerably smaller than observed in patients with cancer, or healthy volunteers, as a result of dilution. This observation may be related to the unusual property of citric acid in solubilizing pro-collagen (Jackson, 1957).

5. *Variations in citrate concentration in blood obtained from various sites*

Citric acid obviously is in a state of rapid flux in the blood, with removal rates in the vicinity of 0·8–0·9 g./hr. which must be matched by release of citrate into blood at an equivalent rate. In an effort to learn more about this phase of citrate metabolism, venous samples were obtained from blood leaving a large mucoid adenocarcinoma of the breast, and compared to mixed venous blood from an antecubital vein :

ABSTRACT

Case No. 1. This Patent, F. W., aged 65, Boston City Hospital No. 1630205, had a 11 × 15 × 15 cm. untreated neoplasm of the left breast reported as mucin secreting adenocarcinoma by biopsy. Two large superficial veins were noted draining the massive primary lesion, passing from the mid left thorax upward to anastomose with communicating branches to the left internal mammary vein. Two citrate tolerance tests were carried out, 1/13/58 and 1/16/58, before and after 30 mg. daily of prednisone therapy, starting 1/14/58 (Table IV). A radical mastectomy on the left was performed on 1/20/58, with 8 negative lymph nodes found. A right radical mastectomy was performed on 1/31/58 for a simultaneous primary on the other side, measuring 1·0 × 1·4 × 1·0 cm., reported as medullary and scirrhus carcinoma, also with negative lymph nodes.

The results demonstrated clearly a decreased citrate concentration in blood leaving this particular cancer, although acid phosphatase activity, which is believed to diffuse from malignant tumours, (Lemon, Davison, and Asimov, 1954), was definitely increased. The latter observation confirms that we were examining a blood sample in the tumour venous bed which differed significantly in its properties from mixed antecubital venous blood, in all six samples. As a result of this observation, some other major source than tumour tissue for citrate diffusion into blood had to be postulated to explain the elevated serum citrate noted in breast and other cancer patients. Simultaneous blood samples from iliac bone marrow, brachial or radial artery, and antecubital vein were then obtained, in a series of healthy volunteers, and cancer patients. A uniform decreasing gradient of citrate concentration from marrow to artery to vein was observed both in representative normal individuals and in cancer patients (Table V).

TABLE V.—*Relative Concentration of Citric Acid in Blood from Various Sites*

Group	No.	Sex	Mean citrate concentration		
			Marrow blood (μg./ml.)	Arterial blood (as % of marrow concentration)	Venous blood (as % of marrow concentration)
Healthy volunteers	11	M.	34·1	89 (4 cases) (range 81–94)	73 (range 51–92)
Metastatic cancer patients (6 under treatment)	8	F.	45·0	93 (range 87–98)	86 (range 67–95)

6. *Serum calcium and phosphorus concentration*

The injection of 0·5 g. of sodium citrate did not significantly change serum calcium, phosphorus or glucose concentration in a sample of 40 patients within the 30 minute period of observation. Hypercalcemia above 12·0 mg. per cent was noted in only one out of 45 patients with active metastatic breast cancer in this study, and in only two out of 85 patients with other types of metastatic cancer. One of the latter cases was a functioning parathyroid carcinoma. The mean serum calcium and phosphorus concentrations for patients sampled within

TABLE VI.—*Calcium Phosphorus and Blood Sugar Observations*

Group	Sex	Number of cases	Venous calcium (mg./100 c.c.)	Venous phosphate (mg./100 c.c.)	Fasting venous blood sugar (mg./100 c.c.)
Healthy volunteers	M.	15	10·2±0·27	2·8±0·08	87·8±0·33
	F.	20	10·1±0·03	3·2±0·19	97 ±4·5 (4 cases)
Rheumatoid arthritis	M.	23	10·1±0·15	3·1±0·12	91·8±4·0
Osteo-arthritis	,,	9	9·7±0·31	2·7±0·15	88·1±2·8
Metastatic carcinoma, breast No therapy	F.	23	10·2±0·48	4·2±0	..
Same, during prednisone therapy:					
Less than 6 months	,,	10	10·1±0·52	3·6±0·28	—
More than 6 months	,,	8	10·0±0·24	3·5±0·21	..
Other metastatic carcinoma No therapy	M.	17	10·4±0·45	3·8±0·08	..
Other metastatic carcinoma No therapy	F.	9	9·9±0·37	3·6±0·04	87·0±8·4

the various groups is shown in Table VI. After an initial group of cancer patients had been sampled, serum calcium concentration was not ascertained except in those who by virtue of extent of metastases or clinical symptoms were considered likely subjects for hypercalcemia. In all these cases, no relationship was ascertainable between the level of serum calcium, and citric acid (Fig. 2). In a patient with a functional metastatic carcinoma of the parathyroid gland, elevated citric acid accompanied hypercalcemia only intermittently.

Metastases of other carcinomas than breast, and sarcomas in both sexes, although sometimes associated with hypercitricemia also failed to show any correlation with serum calcium or phosphorus concentration (Fig. 2).

7. *Hypercitricemia and site of metastases*

The major sites of metastases were compiled in patients showing hypercitricemia, which in some patients constituted at least two major areas (Table VII). Not only was hypercitricemia noted in the absence of clinically demonstrable metastasis, but it was noted in association with each of the major areas of metastasis. Extensive osteolytic activity was present in several patients with metastatic breast cancer, or multiple myeloma without hypercitricemia. However, these patients were receiving some type of antitumour therapy at the time of observation. Major hepatic involvement, which might affect citrate metabolism, was present in only one-fourth of patients with hypercitricemia; and adrenal metastases were uncommon. All the major pathologic types of cancer thus far investigated may induce hypercitricemia sooner or later.

8. *Hypercitricemia and cancer therapy*

In our preliminary report it was emphasized that hypercitricemia was encountered far more often in patients whose cancers had not received any recent antitumour therapy (Lemon, Mueller, Looney, Chasen and Kelman, 1959). A more extensive analysis by cases indicates a substantially similar picture, with the highest frequency (55 per cent) of abnormal citrate values in patients prior to any

form of anti-cancer treatment (Table VIII). The type of therapy utilized differed, in that while most of the breast cases were receiving prednisone, patients with cancer arising from other sites were receiving radiation or analgesic drug therapy only. In several patients, serial observations showed a steady rise in venous citrate concentration in their terminal stages of cancer, in spite of therapy which was obviously ineffective.

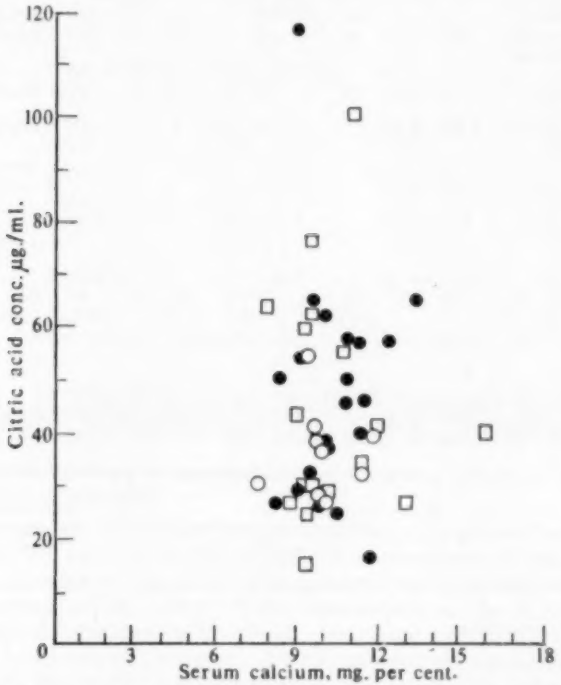


FIG. 2.—Scatter diagrams of relationship between serum citrate and calcium concentration in 47 patients with neoplastic disease. Upper normal citrate concentration for males 41 μg/ml., females 62 μg/ml. The highest serum calcium concentration was seen in a male with a functioning metastatic parathyroid carcinoma.

● 21 ♀, Metastatic carcinoma of the breast.
○ 9 ♀, Non-breast metastatic carcinoma.
□ 17 ♂, Non-breast metastatic carcinoma.

9. *Hypercitricemia and serum enzymatic hyperactivity*

The battery of serum enzyme analysis was not extended to the arthritics or healthy volunteers included in this project since ample data was available in the literature concerning the " normal " values for these disease parameters, and also from our own studies of other groups of patients (Reynolds, Lemon and Byrnes, 1956). Copper-resistant acid phosphatase was elevated above normal in venous blood in the majority of the untreated breast cancer patients, and tended to

TABLE VII.—*Location of Principal Metastases in Patients with Hypercitricemia*

Pathological diagnosis of neoplasm	Number of patients	Location of metastases: Local recurrence and/or lymphatic	Lung, pleura, mediastinium	Liver, portal area	Bone	Remarks
A. Sarcoma	3	1	2	—	1	Adrenals (1).
B. Epidermoid and undifferentiated carcinoma	13	4	4	2	2	Adrenals (1).
C. Adenocarcinoma (non-breast)	8	5	3	3	1	Brain (1).
D. Adenocarcinoma of breast	16	—	3	5	5	Brain (2).
E. Other	5	—	—	—	—	No metastases in carcinoma of bladder (1), adenocarcinoma of colon (2), renal cell carcinoma (1), basal cell (1).
Total patients with hypercitricemia (initial observations)	45	10 (22%)	12 (27%)	10 (22%)	9 (20%)	(No metastases present in 10%).

TABLE VIII.—*Relation of Anti-cancer Therapy to Occurrence of Venous Hypercitricemia in* 101 *Patients with Various Types of Cancer*

Source of cancer	Frequency of hypercitricemia*: No anti-cancer therapy during observation — Total cases	No anti-cancer therapy during observation — Elevated (*)	Anti-cancer therapy concurrently or within past 6 months — Total cases	Anti-cancer therapy concurrently or within past 6 months — Elevated (*)
Breast	18	9	28	11
Other genito-urinary	4	3	13	5
Respiratory	8	7	11	4
Gastro-intestinal	6	2	3	2
Mesenchymal	5	2	5	1
Totals	41	23 (55%)	60	23 (38%)

* $> 2 \times$ S.D. of normal mean venous citrate level (95 per cent level of confidence).

increase with time, especially during the second six months of prednisone therapy (Table IX). Phosphohexose isomerase analyses showed a similar trend. The data are insufficient to draw conclusions from either alkaline phosphatase or transaminase measurements. Citrate concentration, however, decreased during the first six months of prednisone treatment of breast cancer, to values well within the normal range, before a later secondary rise, suggesting different factors influenced venous concentration of the latter during treatment compared to various enzymatic functions. Other types of metastatic cancer in men and women also often had abnormal acid phosphatase and phosphohexose iseromase activity as shown by the mean values, although the variation of activity from case to

TABLE IX.—*Enzyme Activity of Venous Blood and Citrate Concentration*

Group	Sex	Alkaline phosphatase (Bodansky units)	Copper resistant acid phosphatase (μmole phenol/100 ml.)	Phosphohexose isomerase (Bodansky units)	Glutamic oxalacetic transaminase (units)	Fasting citrate concentration (μg./ml.)
Normal . . .	M.	Under 5	Under 24 μmole/100 ml.	Under 40	Under 20 units	27·2±1·3 (27)
	F.	..	..	..	..	38·3±1·8 (44)
Metastatic carcinoma of breast No therapy	„	5·95±1·13 S.E. (19)	38·8±4·2 S.E. (27)	42·6 (22)	17·5 (8)	45·2±3·8 S.E. (29)
Same, prednisone therapy 6 months or less		7·3 (3)	40·1±6·9 (13)	69·2±18·6 (10)	15 (4)	33·6±2·9 (13)
Same, prednisone for over 6 months		14·6 (5)	46·8±4·9 (10)	63·3±52 (9)	35·8 (4)	44·3±5·9 (12)
Other metastatic carcinoma No therapy	M.	5·8±1·7 (12)	33·3±5·6 (19)	41·6±5·6 (14)	12 (3)	43·9±3·8 (25)
Other metastic carcinoma No therapy	F.	8·2±3·2 (6)	32·8±4·6 (10)	53·4±14·5 (10)	12·7 (3)	39·9±5·0 (15)

Numbers in parentheses indicate number of patients tested, upon which mean ± S.E. is based.

case was great. In this latter group, enzymatic abnormalities also appeared to vary independently of citrate concentration in many individual cases.

10. *Reduction of elevated citrate concentration in mammary cancer by anti-estrogenic therapy with prednisone*

During these studies, hormonal therapy consisting of oophorectomy in patients under 64 years of age, combined with pituitary-adrenocortical suppression utilizing prednisone 20–30 mg. daily was given to many of the new advanced breast cancer cases (Lemon, 1959). Serial observations of the immediate effects of prednisone administration upon citrate dynamics were made in 7 healthy volunteers and in 10 cancer patients. An inconsistent change in fasting venous citrate was noted in healthy volunteers receiving prednisone, while the cancer patients with hypercitricemia (including one with bronchogenic carcinoma) in nearly all cases had a significant ($p = < \cdot 01$) reduction of citrate concentration to normal female or even normal male values during the first 6 months of treatment (Table IX). This fall in venous citric acid concentration was accompanied by a mean 28 per cent reduction in 18 estimations of the clearance rate of injected citric acid in chiefly female cancer patients, whose gonads had been ablated (Tables III, X). The less regular reduction of citrate clearance rate was noted in healthy volunteers, whose intact gonads possibly partially nullified the anti-estrogenic effects of prednisone therapy, and whose citrate concentrations were initially within the normal range. These observations appear consistent with a cortisone inhibition of citrate diffusion into blood in cancer patients, leading to a reduction of blood citrate concentration in spite of reduced citrate uptake by tissues.

It was striking to note the recurrently elevated blood citrate values when patients relapsed while on adrenal corticoid therapy (Table XI).

TABLE X.—*Effect of Prednisone Therapy on Citrate Clearance (C) in Volunteers and Cancer Patients*

Healthy volunteers (Male)	Duration therapy	Per cent change from pre-therapy observation	Cancer patients (Male)	Origin	Duration therapy	Per cent change from pre-therapy observation
J. L—	3 days	+55	F. C—	Lung	8 days	−70
J. S—	,,	+75				
B. I—	,,	−12				
R. N—	,,	−37	(Female)			
			E. W—	Breast	1 day	+31
(Female)			I. G—	Endometrium	27 days	−63
L. K—	,,	−11	H. A—	Breast	2 days	−13
R. D—	,,	−36	,,	,,	6 weeks	+22
V. D—	,,	−62	,,	,,	2½ months	−41
			P. I—	,,	6 days	−61
			,,	,,	2 months	−43
			,,	,,	5 months	+18
			,,	,,	6 months	+31
(Dosage = 30 mg./day)			B. L—	,,	9 days	−46
			B. T—	,,	11 days	−67
			L. M—	,,	9 months	+ 3
			,,	,,	1 year	−24
			A. M—	,,	12 days	−14
			M. C—	,,	6 months	−90
			M. L—	,,	9 days	−60
			,,	,,	2 weeks	−22
7 patients		Mean = −4% (7 tests)	11 patients			Mean = −28% (18 tests)

TABLE XI.—*Recurrence of Hypercitricemia During Clinical Relapse of Advanced Mammary Cancer Treated by Prednisone*

Patient	Metastases	Initial venous citrate (μg./ml.)	Mean, range of venous citrate duration theraputic remission	Duration therapy	Venous citrate during terminal relapse (therapy continued) (μg./ml.)	Time before death
H. A—	Bone, liver later	64·8	30·5 (23·1–37·2)	6 months	62·3	1 month
B. L—	Bone, lung	64·8	49·4 (47·4–51·4)	1 month	103·0	2 weeks
H. C—	Brain, lung	100+	20·2 (18·5–21·9)	2 months	100+	1 month
K. L—	Lung, mediastinum	69·5	50·0	2 months	30·4	1 month
L. M—	Skin, bone, liver later	50	34·8 (27·4–41·9)*	1 year	76·1*	2 months
		..	34·1 (30·2–38·0)	..	116·6	1 week
L. T—	Bone, lung	79·0	30·1 (18·8–40·3)	18 months	23·9	1 month
P. I—	Bone	57·2	29·4 (19·4–39·1)	7 months	75·9 (62–95·5)	3 months
F. C—	,,	89·5	34·5 (22·2–49·7)	18 months	47·9 (26·9–64)	7 months
M. L—	,,	100+	39·4 (21·6–60·8)*	2 years	79·2 (66–106)*	..
			31·5 (16·3–39·1)	..	39·1	(Living)
Mean of patients means :		75	35·0	..	65·9	..

* Temporary relapse.

DISCUSSION

Hypercitricemia in various types of cancer has not been frequently noted by several previous observers (Schersten, 1931; Rottino, Hoffman and Brondolo, 1952) with a single undocumented exception (Kyle and Canary, 1957). Review of the case material utilized in several of these papers indicates that only a small number of patients were studied, of whom many had already received anti-cancer therapy. Furthermore, it is not clear whether fasting bloods were always utilized for analyses, which will usually yield the peak venous citrate concentrations. Relatively few case reports are included of breast, prostate and lung carcinomata which comprise the majority of our patients with hypercitricemia. Simultaneous pre-selected control groups were not used in some previous studies, retrospective controls being used. These differences appear to explain most of the discrepancies between our observations, and previous reports.

In spite of inflammatory disease involving bone and joints in rheumatoid arthritis and metabolic disorders, such as post-menopause osteoporosis, serum citrate was normal in all non-cancer cases. Atrophic bone changes were frequently widespread as shown by X-ray at the time of our sampling. This suggests that citrate measurement can be a useful adjunct in differential diagnosis of the benign or malignant nature of some osseous lesions, in which demineralization is a prominent feature.

From our observations hypercitricemia appears to be a relatively common disorder usually independent of hypercalcemia in patients with advancing neoplastic disease invading liver, lung or bone among other tissues. Hypercitricemia during active cancer growth is of particular interest in that it may represent a dysfunction of the Krebs cycle through excessive citrate production or deficient citrate utilization via condensation to acetoacetate, either in cancer or normal tissues or both. Destruction of the activity of Coenzyme II (TPN) might result in such a disturbance. In addition citrate equilibrium in osseous tissues may be disturbed, as seen in hyperparathyroidism or Paget's disease with hypercitricemia secondary to osteolysis (Kissin and Kreeger, 1954; Chang and Freeman, 1950*b*). In this latter case, a negative calcium balance and hypercalcemia might be expected to accompany hypercitricemia, as we have observed in one case of functioning parathyroid carcinoma and in occasional mammary cancer patients. Intensive osseous destruction, although present roentgenologically in many of the hypercitricemia breast cancer patients, was not severe enough to induce hypercalcemia in most of these patients and may be coincidental, rather than contributory.

Gomori and Gulyas (1944) were the first to observe that parenteral administration of sodium citrate in dogs leads to marked hypercalcuria with minimal alteration in serum calcium concentration. Their work has been confirmed (Chang and Freeman, 1950*a*), indicating that in the absence of hyperparathyroidism and its attendant metabolic disturbance hypercitricemia may produce a ten to twenty fold augmentation of calcium excretion without raising total serum calcium concentration above 12 mg. per cent. This may be the result of a marked increase in the ultra-filterable fraction of plasma calcium which is citrate bound, or because of diminished tubular reabsorption of the calcium citrate complex. These observations however help to explain the rare coexistance of hypercitricemia and hypercalcemia in our cancer population.

Hormonal factors such as insulin (Pincus, Natelson and Lugovoy, 1949), epinephrine (Pincus, Natelson and Lugovoy, 1951) and adrenocortical hormones (Pincus, Natelson and Lugovoy, 1951; Agrell, Lindell and Westling, 1955) also have been shown to alter venous citrate concentration. None of our hypercitricemic cancer patients was diabetic or receiving insulin, nor did we encounter any pheochromocytomas in our series of cases. Although adrenocortical insufficiency results in hypercitricemia in man (Martenesson, 1949), none of our patients was suffering from acute adrenocortical insufficiency at the time of our studies, and only one patient with leiomyosarcoma metastatic to the adrenal glands was receiving adrenal steroid therapy, as a result of a previous Addisonian crisis. At the time of our study this patient was in electrolyte balance. Although the kidneys serve as a major site of citrate uptake from blood, significant impairment of renal function was not present in our hypercitricemia patients. Hepatic insufficiency was also absent in most hypercitricemia patients, including those with hepatic metastases. No evidence was obtained that hypercitricemia was related to any reduction of citrate uptake from blood (Table III).

The striking reduction of venous citrate concentrations to normal in previously hypercitricemia breast cancer patients receiving cortisone or prednisone, occurred in spite of a 28 per cent reduction in clearance rate for injected citrate. This depression of citrate uptake from blood by adrenal steroid therapy in all likelihood is closely related to the reduction of acetate utilization which has also been reported. Hennes and Shreeve administered doses of prednisone identical to those we have utilized, to patients receiving ^{14}C-labelled acetate, and noted an initial reduction of 20–30 per cent in rate of radiocarbon excretion compared to control observations (Hennes and Shreeve, 1959). Over a 24 hour period a 10–15 per cent reduction of cumulative radioactivity excretion was demonstrated. Henneman and Bunker have reported elevated venous lactate and pyruvate concentrations following adrenal steroid therapy and in Cushings' syndrome (Henneman and Bunker, 1957), suggesting a decrease in pyruvate oxidation under these circumstances. Impairment of glucose tolerance has long been recognized as one of the most dependable laboratory manifestations of adrenal cortical hyperfunction.

The control of hypercitricemia by adrenal steroid therapy must be accounted for by reduced citrate diffusion into blood from some tissue source. Not only must this diffusion rate be very high prior to therapy to induce hypercitricemia, in view of the 20–25 g./day capacity of the body to utilize citrate, but steroid induced inhibition of diffusion from this tissue source must far exceed the net overall reduction in uptake of citrate caused by prednisone therapy. Our observations indicate that a major source of citrate enrichment of blood exists in the sinusoids of bone marrow, where at all times citrate concentration exceeds that of mixed peripheral arterial or venous blood. Adrenal steroid therapy appears to have a more marked and consistent effect reducing venous citrate of breast cancer patients than that of healthy volunteers. However, differences in the duration and intensity of therapy or in the degree of sex hormone inhibition so induced, may account for this variation in response. The greater prevalence of hypercitricemia in advanced breast, prostate and lung carcinoma patients in whom osseous metastases are so common (Table III) and the infrequency of occurrence in benign tumors or localized breast cancer suggest that hypercitricemia is potentiated by widespread neoplastic invasion of bone marrow. The

simultaneous elevation of venous acid phosphatase in hypercitricemic patients also supports bone marrow invasion as the chief source for excessive diffusion of citrate into blood, since this enzyme is elevated in venous blood in 75 per cent of patients with osseous metastases of breast or prostate carcinoma prior to therapy (Reynolds, Lemon and Byrnes, 1956). We have also found that marrow sinusoid blood is far higher in acid phosphatase activity, than peripheral arterial or venous blood (Reynolds, Lemon, Kaplan, Idelson, Mueller and Derow, 1959). Likewise, abnormal venous phosphohexose isomerase activity is often present in mammary carcinoma with osseous invasion (Bodansky, 1954*a*), and elevated alkaline phosphatase activity is well known to result from osseous or hepatic invasion by tumour.

Since the early reports of Dickens and others concerning the presence of citrate in the organic matrix of vertebrate bone (Dickens, 1941 ; Thunberg, 1953), which contains 95 per cent of total body citrate, a great deal of work has been carried out showing a close relationship between calcium and citrate metabolism in response to various stimuli, and a current hypothesis of bone formation includes precipitation of calcium citrate on the superficial lamellae of bone trabeculae (Neumann and Neumann, 1958). Enzymes necessary for local production of citrate have been described in osteoid tissue (Dixon and Perkins, 1952). One observed case of osteogenesis imperfecta, in a 5 year old boy, had a venous citrate of 89·5 μg./ml. prior to therapy, falling to a normal value after testosterone therapy had induced some calcification and clinical improvement. The movement of calcium and citrate in and out of bone under the influence of parathormone or Vitamin D therapy is generally in the same direction (Carlson and Hollunger, 1954 ; Elliott and Freeman, 1956), and a similar trend is apparent in our data. With the exception of prostatic carcinoma which rarely induces hypercalcemia or hypercalcuria, cancer frequently resulting in hypercitricemia such as lung or breast are also prone to develop hypercalcemia. When the latter develops, hypercitricemia usually co-exists. The " Idiopathic " hypercalcemia reported in advanced lung, breast, ovarian or renal cancer cases in the absence of detectable osseous metastases may be possibly associated with hypercitricemia, if the latter abnormality were to be looked for (Plimpton and Gellhorn, 1956). A calcium-binding substance has been postulated in those cases in whom an elevated serum alkaline phosphatase suggested bone disease.

Normal prostatic and mammary epithelium secrete extremely high concentrations of citric acid into their respective secretions as a result of specific sex hormonal stimulation (Mann, 1954 ; Lenner, 1934) and this function possibly is preserved in some endocrine dependent cancers. Talalay and Williams-Ashman have postulated that estrogenic cellular stimulation is mediated via effects upon the balance of pyridine nucleotide dependent transdehydrogenase systems involved in the function of the Krebs cycle (Talalay and Williams-Ashman, 1958), which would therefore govern the production and/or utilization of citrate by hormone sensitive tissues.

Our one negative observation of citrate diffusion from breast cancer occurred in a patient with primary disease lacking demonstrable lymphatic or osseous metastases and whose venous blood levels were normal. If tumor cells in bone marrow served as a major source for the high citrate concentrations we have noted, osteolytic bone destruction might be explained by the reversal of the normal processes of calcification, through creation of excessive amounts of free citrate in

the diseased sinusoids to compete with phosphate for the calcium hydroxyapatite (Neumann and Neumann, 1958). Neoplastic invasion of soft tissues may also be facilitated by citrate induced solvation of procollagen (Jackson, 1957).

Numerous observations attest to the striking ability of cortisone and prednisone therapy to control hypercalcemia in cancer, and to induce recalcification of osseous metastases in breast cancer, coincidental with reduction of circulating citrate concentration to normal (Lemon, 1956, 1957, 1959; Nissen-Meyer, 1957). Adrenal corticoids have been shown to antagonize the action of estrogens upon hormone-dependent target tissues such as endometrium, nullifying both water inhibition and growth (Szego and Roberts, 1953; Huggins and Jenson, 1955; Velardo, Hisaw and Bever, 1956) as well as exerting a depressing influence upon the growth of a number of different types of transplantable and spontaneous cancers (Pearson, Li, MacLean, Lipsett, and West, 1955; Rusch, 1956). As a working hypothesis it may be postulated that the normalization of venous citrate and subsequent recalcification of some osseous metastases may also represent the result of direct inhibitory effects exerted upon osseous mammary carcinoma metastases as well as indirect effects secondary to depressed sex hormone secretion. Our data is insufficient as yet concerning the influence of steroid therapy upon hypercitricemia in other types of metastatic cancer to draw further inferences. Although clinically obvious metastases were noted in hepatic or pulmonary or other areas without radiologically detectable bone involvement in 71 per cent of our hypercitricemia cases with metastatic cancer (Table VII) the prevalence of circulating tumour emboli in over 50 per cent of patients with malignant neoplasms insures that the sinusoids of bone marrow become the repository of active or inactive metastases, sooner or later in most forms of advanced cancer (Fisher and Turnbull, 1955; Moore, Sandberg and Schubarg, 1957; Engell, 1955).

Finally, hypercitricemia to the levels which we have observed must produce some major disturbances of membrane permeability, resulting from alteration of the mono-valent to di-valent cation ratio through calcium binding by elevated citrate. Normally female venous blood contains about 0·6 m-equiv./l. of citrate (38 μg./ml.) capable of binding a similar amount of ionized calcium (Hastings, MacLean, Eichelberger, Hall and Da Costa, 1934). About 65 per cent of serum calcium is normally ultra-filterable or 3·24 m-equiv./l. (Neumann and Neumann, 1958). Bicarbonate, phosphate and citrate are the principal anions available to bind diffusible calcium. Under normal circumstances, ionized calcium averages about 2·66 m-equiv./l., so that citrate bound serum calcium constitutes a labile fraction amounting to nearly one-sixth of total diffusible calcium. If citrate concentration rises to the values herein reported, then 1·0–1·8 m-equiv./l. or more of serum calcium ion might be complexed with citrate, assuming no shift of the latter from protein, leaving only an estimated 1–2 m-equiv./l. of ionized calcium for control of membrane permeability. Hastings and co-workers clearly showed that calcium citrate was metabolically inert in so far as the frog heart was concerned, up to values as high as 20 m-mole/l. (Hastings, MacLean, Eichelberger, Hall and Da Costa, 1934), and hence cannot function in the control of membrane permeability.

Contrary to some earlier reports (Allen, Clark, Thornton and Adams, 1944) hypercitricemia has been observed as a hazardous complication of exchange transfusion in infants (Wexler, Pincus, Natelson and Lugovoy, 1949), and of

massive blood replacement therapy in adults with hepatic cirrhosis and bleeding varices, major vascular surgery and major operations in the portal area (Bunker, Stetson, Coe, Grillo and Murphy, 1955). Bunker and co-workers observed that the immediate rise in serum citrate after infusion of varying amounts of citrated blood varied with the rate of administration and total amount of blood infused. Somewhat higher arterial blood levels were observed in patients with hepatic disease, than in other pre-operative patients under pre-medication. Values were noted as high as 300–400 μg./ml. in their series (± 5·0–6·5 m-equiv./l.). Depression of ionic calcium concentration calculated on the basis of total calcium, total protein and citrate concentration (Hastings, MacLean, Eichelberger, Hall and Da Costa, 1934) to values as low as 1·10–1·40 m-equiv./l. was associated with hypotension or shock in the majority of cases, a few of whom responded to intravenous calcium ion therapy. Only rarely did their patients show tetanic manifestations which we have not observed as yet, either. With calculated ionic calcium above 1·50 m-equiv./l., no hypotensive phenomena were observed. Less deleterious subjective effects may result from smaller elevations of serum citrate such as we have noted, which consisted primarily of anorexia and nausea, and emphasize that hypercitricemia may contribute to malaise of the cancerous patient. Reduction of blood citrate levels to normal by prednisone therapy, and the simultaneous striking subjective improvement of many patients, including their strength and appetite, may be related to a restoration of a more normal ionic cellular environment.

In most of the adult surgical patients in whom massive replacement of blood is necessary, extreme activation of the pituitary-adrenocortical system by the underlying catastrophic disease probably contributed to hypercitricemia, by impairing the metabolism of exogenous citrate, such as we have observed occurs after adrenal cortical steroid therapy.

Cookson and co-workers have suggested that hypothermia which has been utilized in vascular surgery may compound the hazard by further reduction of ability to utilize large amounts of administered citrate (Cookson, Costas-Durieux and Bailey, 1954). Anoxia *per se* may also induce hypercitricemia (Hallman and Forsander, 1952).

Bunker and co-workers obtained fairly satisfactory agreement between the observed increment in arterial citrate concentration after infusion (average in 11 patients=13·7 mg./100 c.c.) and the increment predicted on the basis of equilibration in extracellular fluid within 6–16 minutes (average=11·2 mg./100 c.c.) If the latter figure is corrected for the mean rate of fall we have noted in serum citrate concentration after termination of infusion, approximately 1 μg./ml./minute in both sexes in the period 5–30 minutes after injection, the expected citrate concentration would be 12·5 mg./100 c.c. from their data, on the assumption that the clearance process begins immediately with initial elevation of serum citrate. It is likely that the amount of protein bound calcium was overestimated from the total protein concentrations observed in many of the patients with hepatic cirrhosis in the series reported by Bunker *et al.* (1955) in whom a considerable increase in gamma globulin fraction could be expected, which has been found to have only slight calcium binding capacity (Carr, 1955). Therefore, their estimates of residual ionic calcium may be somewhat on the low side. Not all recent investigators have found hypercitricemia after massive transfusion, no doubt due partly to the very rapid utilization of citrate which all have noted,

even with advanced liver disease (Howland, Bellville, Zucker, Boyan and Cliffton, 1957).

SUMMARY

Citric acid concentration has been measured using the pentobromoacetone procedure in blood from bone marrow, arterial and venous sites, in 358 healthy volunteers, arthritics and patients with various types of cancer in all stages. The clearance of exogenous citrate from blood has been estimated in a representative fraction of each group of patients and approximates 0·9 g. per hour. Women have been found to have consistently higher fasting venous serum citric acid than men, with a slightly lower citrate clearance rate. Bone marrow blood consistently shows the highest concentration of citrate, followed by arterial and finally venous blood. Venous citric acid concentrations greater than twice the S.D. from the mean for each sex ("hypercitricemia") occurred in 4·2 per cent of healthy volunteers, in 4·6 per cent of all patients with benign diseases, in 11·1 per cent of patients with pre-malignant tumors, and in 23·6 per cent of patients with cancer. Anorexia and weakness were generally noted in the latter group. Hypercalcemia exceeding 12 mg. per cent (6 m-equiv./l.) was noted in only 3 patients in the entire series, but abnormal acid phosphatase and lactic dehydrogenase activity usually accompanied hypercitricemia. Advanced carcinomas of the breast, prostate, and lung were most commonly associated with hypercitricemia, probably as a result of osseous metastases. Prednisone therapy in advanced mammary carcinoma usually reduced elevated blood citric acid to normal simultaneously reducing by 28 per cent the utilization of administered citrate, indicating the probable suppression of tumor growth in osseous and perhaps in other metastatic sites.

These studies have been supported by a research grant from the National Cancer Institute, by the Rebecca Rice Memorial Grant of the American Cancer Society to Boston University, by a grant from the Quincy United Fund, of Quincy, Massachusetts, and a student research fellowship from the Massachusetts Arthritis and Rheumatism Foundation.

REFERENCES

Agrell, I., Lindell, S. E. and Westling, H.—(1955) *Acta physiol. scand.*, **34**, 135.
Allen, J. G., Clark, D. E., Thornton, T. F. and Adams, W. E.—(1944) *Surgery*, **15**, 824.
Bodansky, A.—(1932) *J. biol. Chem.*, **99**, 197
Bodansky, O.—(1954*a*) *Cancer*, **7**, 1191.—(1954*b*) *Ibid.*, **7**, 1200.
Bunker, J. P., Stetson, J. B., Coe, R. C., Grillo, H. C. and Murphy, H. D.—(1955) *J. Amer. med. Ass.*, **157**, 1361.
Carlson, A. and Hollunger, G.—(1954) *Acta physiol. scand.*, **31**, 317.
Carr, C. W.—(1955) in 'Electrochemistry in Biology and Medicine', Shedlovsky, T. (ed.). Chapter 14. New York (John Wiley and Sons).
Chang, T. S. and Freeman, S.—(1950*a*) *Amer. J. Physiol.*, **160**, 330.—(1950*b*) *Ibid.*, **160**, 341.
Clark, E. P. and Collip, J. B.—(1925) *J. biol. Chem.*, **63**, 461.
Cookson, B. A., Costas-Durieux, J. and Bailey, C. P.—(1954) *Ann. Surg.*, **139**, 430.
Cori, C. F. and Cori, G. T.—(1925) *J. biol. Chem.*, **65**, 397.

DICKENS, F.—(1941) *Biochem. J.*, **35**, 1101.
DIETRICH, L. A. AND SHAPIRO, D. M.—(1956) *Cancer Res.*, **16**, 585.
DIXON, T. F. AND PERKINS, H. R.—(1952) *Biochem. J.*, **52**, 260.
ELLIOTT, J. R. AND FREEMAN, S.—(1956) *Endocrinology*, **59**, 200.
ENGELL, H. C.—(1955) *Acta chir. scand.*, Supp. 201, 1.
ETTINGER, R. H., GOLDBAUM, L. R., SMITH, L. H.—(1952) *J. biol. Chem.*, **199**, 531.
FISHER, E. R. AND TURNBULL, R. B.—(1955) *Surg. Gynec. Obstet.*, **100**, 102.
FISKE, C. H. AND SUBBAROW, Y.—(1925) *J. biol. Chem.*, **66**, 375.
FRANCO.—(1957) 'Sigmo-Franco Procedure', *Sigma Chem. Co. Tech. Bulletin*, No. 505.
GOMORI, G. AND GULYAS, E.—(1944) *Proc. Soc. exp. Biol. N.Y.*, **56**, 226.
HASTINGS, A. B., MACLEAN, F. C., EICHELBERGER, L., HALL, J. L. AND DA COSTA, E. —(1934) *J. biol. Chem.*, **107**, 351.
HALLMAN, N. AND FORSANDER, O.—(1952) *Ann. Med. exp. Fenn.*, **30**, 287.
HENNEMAN, D. H. AND BUNKER, J. P.—(1957) *Amer. J. Med.*, **23**, 34.
HENNES, A. R. AND SHREEVE, W. W.—(1959) *Proc. Soc. exp. Biol. N.Y.*, **100**, 246.
HERNDON, R. F. AND FREEMAN, S.—(1958) *Amer. J. Physiol.*, **192**, 369.
HILL, B. R. AND LEVI, C.—(1954) *Cancer Res.*, **14**, 513.
HOWLAND, W. S., BELLVILLE, J. W., ZUCKER, M. B., BOYAN, P. AND CLIFFTON, E.— (1957) *Surg. Gynec. Obstet.*, **105**, 529.
HUGGINS, C. AND JENSON, E. V.—(1955) *J. exp. Med.*, **102**, 347.
JACKSON, D. S.—(1957) 'Connective Tissue. A Symposium'. Tunbridge *et al.* (ed.). Oxford (Blackwell Scientific Publications), p. 62.
KISSIN, B. AND KREEGER, N.—(1954) *Amer. J. med. Sci.*, **228**, 301.
KYLE, L. H. AND CANARY, J. J.—(1957) *J. Lab. clin. Med.*, **49**, 590.
LEMON, H. M.—(1956) *Clin. Congr. Amer. Coll. Surg.*, **6**, 415.—(1957) *Annals intern. Med.*, **46**, 457.—(1959) *Cancer*, **12**, 93.
Idem, DAVISON, M. D. AND ASIMOV, I.—(1954) *Ibid.*, **7**, 92.
Idem, MUELLER, J. H., LOONEY, J. M. CHASEN, W. H. AND KELMAN, M.—(1959) *Boston med. quart.*, **10**, 1.
LENNER, A.—(1934) *Acta obstet. gynec. scand., Supp.* 1 ; **14**, 1.
MANN, T.—(1954) 'The Biochemistry of Semen'. London (Methuen and Co., Ltd.), p. 16.
MARKS, P. A. AND BISHOP, J. S.—(1957) *J. clin. Invest.*, **36**, 254.
MARTENSSON, J.—(1949) *Acta med. scand.*, **134**, 61.
MILLER, H. AND CARRUTHERS, C.—(1950) *Cancer Res.*, **10**, 636.
MOORE, G. E., SANDBERG, A. AND SCHUBARG, J. R.—(1957) *Ann. Surg.*, **146**, 580.
NEUMANN, W. F. AND NEUMANN, M. W.—(1958) 'The Chemical Dynamics of Bone Mineral'. (Univ. of Chicago Press), p. 209.
NISSEN-MEYER, R.—(1957) *Acta endocr., Supp.* 31, p. 314.
PEARSON, O. H., LI, M. C., MACLEAN, J. P., LIPSETT, M. B. AND WEST, C. D.—(1955) *Ann. N.Y. Acad. Sci.*, **61**, 393.
PINCUS, J. B., NATELSON, S. AND LUGOVOY, I. K.—(1949) *J. clin. Invest.*, **28**, 741.— (1951) *Proc. Soc. exp. Biol. N.Y.*, **78**, 452.
PLIMPTON, C. H. AND GELLHORN, A.—(1956) *Amer. J. Med.*, **21**, 750.
POTTER, V. R. AND BUSCH, H.—(1950) *Cancer Res.*, **10**, 353.
RECHENBERGER, J. AND BENNDORF, S.—(1956) *Z. Altersforsch.*, **10**, 49.
REYNOLDS, M. D., LEMON, H. M. AND BYRNES, W. W.—(1956) *Cancer Res.*, **16**, 943.
Idem, LEMON, H. M., KAFLAN, G. A., IDELSON, B. A., MUELLER, J. AND DEROW, M. A. —(1959) *Proc. Amer. Ass. Cancer Res.*, **3**, 56.
ROTTINO, A., HOFFMAN, G. T. AND BRONDOLO, B.—(1952) *Proc. Soc. exp. Biol. N.Y.*, **80**, 339.
RUSCH, H. P.—(1956) *Cancer Res.*, **16**, Supp. 4, p. 183.
SAFFRON, M. AND DENDSTADT, O. F.—(1948) *J. biol. Chem.*, **175**, 849.
SCHERSTEN, B.—(1931) *Skand. Arch. Physiol.*, **63**, 97.

SCHWARTZ, M. K., GREENBERG, E. AND BODANSKY, O.—(1959) *Proc. Amer. Ass. Cancer Res.*, **3**, 61.
SIBLEY, J. A., FLEISCHER, G. A., HIGGINS, G. M.—(1955) *Cancer Res.*, **15**, 306.
STARK, J. B., GOODBAR, A. E. AND OWENS, H. S.—(1951) *Analyt. Chem.*, **22**, 413.
SZEGO, C. M. AND ROBERTS, S.—(1953) *Recent Progr. Horm. Res.*, **8**, 419.
TALALAY, P. AND WILLIAMS-ASHMAN, H. G.—(1958) *Proc. nat. Acad. Sci., Wash.*, **44**, 15.
THUNBERG, T.—(1953) *Physiol. Rev.*, **33**, 1.
VELARDO, J. T., HISAW, F. L., BEVER, A. T.—(1956) *Endocrinology*, **59**, 165.
WEXLER, I. B., PINCUS, J. B., NATELSON, S. AND LUGOVOY, I. K.—(1949) *J. clin. Invest.*, **28**, 474.
WOLFSON, S. K., SPENCER, J. A., STERKEL, R. L. AND WILLIAM-ASHMAN, H. G.—(1958) *Ann. N.Y. Acad. Sci.*, **75**, 260.
YARBO, C. L.—(1956) *J. Urol.*, **75**, 216.

SURVEY OF PAPERS

Boddington, Cowdell and Spriggs have reviewed 10,000 cervical smears from 8522 patients and have tried to correlate the cytological abnormalities found with the associated histological changes and with subsequent developments.

They find that epithelial instability (basal cell hyperplasia, "carcinoma *in situ*" and related lesions) usually disappears without any treatment more extensive than biopsy, but may become irreversible, in which case invasive carcinoma sooner or later develops without any cytological warning (p. 151).

Martins reports an adenocarcinoma of the uterus in a year old child (p. 165).

Holland, Acevedo and Clark find that the mean arsenic content of the bronchial mucosa and submucosa of lung cancer victims is significantly higher than the mean value found in controls (p. 169).

Baserga, Putong, Tyler and Wartman, using Ehrlich ascites tumour cells and $CxAF_1$ mice, report experiments planned to establish a quantitative relationship between the number of embolic tumour cells and the incidence of lung metastases. At the same time they investigated other factors said to affect the incidence of blood-borne metastases such as the sex of the animal, simultaneous injection of killed tumour cells, pre-treatment with viable tumour cells, as well as the activity of the reticulo-endothelial system (p. 173).

Hewitt and Wilson report experiments undertaken with the object of examining various factors which could affect proper interpretation of their previously described survival curve for CBA mouse leukaemia cells irradiated *in vivo* in mice of the substrain of origin (p. 186).

F. Bielschowsky and M. Bielschowsky find that the tumours that develop in pituitary dwarf mice after treatment with 2-aminofluorene differ in their distribution from that seen in their normal sized litter mates similarly treated (p. 195).

Pearson describes the histological and cytological changes in serially irradiated tumours in RIII inbred mice and discusses the significance of the changes in relation to radiosensitivity, cell degeneration and invasive properties (p. 200).

Israel and Ellis have administered methylthiouracil to mice on normal and low iodine diets. They describe the hyperplastic changes which resulted in the thyroids of the mice and emphasise the extreme difficulty in producing thyroid neoplasms in mice, even under the most intense stimulation (p. 206).

Gradially gives an account of the tumours which resulted from repeated applications of 9 : 10 dimethyl-1 : 2 benzanthracene to the skin of a hedgehog. The growths were essentially similar to those seen in the skin of man and laboratory rodents (p. 212).

Louis finds that the fluorescein-globulin affinities of normal, hyperplastic and neoplastic cells of the virus induced Shope papilloma are identical to those of chemically induced and naturally occurring neoplasms (p. 216).

Belcher and Simpson, using ^{51}Cr- and ^{59}Fe-techniques, have investigated the acute haemolytic episode which occurs in rats of the August strain 10–15 days after implantation of a transplantable mammary adenocarcinoma and also in normal animals transfused with blood from tumour-bearing litter-mate donors (p. 224).

Miller has shown that thymus tissue from genetically susceptible mice can undergo malignant transformation in a foreign but tolerant host, and that a number of such malignant thymuses can be made to regress completely following the inoculation of activated immunologically competent cells (p. 244).

Mody gives an account of the development of ovarian tumours in inbred virgin IF mice following limited skin applications of carcinogenic hydrocarbons (p. 256).

Pullinger and Iversen record and analyse quantitatively, for subsequent comparisons, reference data in respect of age, number of litters and mammary carcinoma incidence in two strains of mice. No conclusion as to the effect of litter number and/or the effect of age upon the incidence of mammary tumours can be drawn (p. 267).

Pullinger describes tests of two strains of mouse for the presence or absence of mammary tumour agent (p. 279).

Young and Frazer have examined histologically over 200 hypophysectomised mice for the presence of residual fragments of pituitary, and have attempted to correlate the presence or absence of such fragments with post-operative changes in the animals' body weight, testis weight, seminal vesicle and adrenal weight, and with the mammogenic growth response after treatment with oestrone and progesterone (p. 285).

Davies and Wilmshurst find that the charred residue of commercial starch "destructively distilled" at atmospheric pressure and at a temperature of 370–390°C. contains 0·2 μg. of benzopyrene per 100 g. "char" (p. 295).

Stein-Werblowsky has applied benzopyrene to the cervix uteri of rats in the dioestrus and oestrus phases. More tumours, including some malignant growths, developed in the animals painted in the dioestrus phase (p. 300).

Drayton describes experiments in which the effect of incubation on the biological activity of three fowl tumour viruses, as determined by bioassay methods, is studied in relation to virus constituents liberated (p. 306).

Allison and Armstrong have followed the progress of infection with polyoma virus of mouse cells in tissue culture. The first change detectable after infection is the appearance of abnormal DNA-containing foci in the nuclei. Later the cells become detached from the coverslips and the nuclei of some are almost filled with the DNA-containing material. They conclude that polyoma virus contains DNA and multiplies in the nuclei of susceptible cells (p. 313).

Montemurro has studied the effect of nitrofurazone on the testes, seminal vesicles and prostate of normal rats and rats bearing the Walker carcinoma 256. He finds that the atrophic changes in the seminal vesicle, and to a lesser degree in the prostate that are associated with the growth of this tumour in rats can be largely prevented by the intraperitoneal administration of nitrofurazone (p. 319).

Clark and Goodlad have studied the effect of the Walker 256 carcinoma on the tissues of host rats receiving diets varying in protein and calorie content. They found tumour growth to be independent of the various dietary treatments employed, and also observed an increase in the protein, RNA and DNA content of the liver of tumour bearing rats, under all dietary conditions (p. 327).

O'Gorman gives a method for demonstrating the cytotoxic action of murine iso-antisera *in vitro*, and describes the effect of iso-antibody on normal leucocytes and several tumours. He discusses the bearing of his findings on the role of circulating antibody in the homograft reaction (p. 335).

Mirvish and Gillman have examined the volume and composition of the bile of rats fed butter yellow. They propose a mechanism involving alterations in the rate of 12-hydroxylation to explain the abnormalities found, and discuss the possible significance of hydroxylations in general for the understanding of cancer (p. 346).

Csaba, Horváth and Ács have investigated the effect of heparin and its components on the survival of mice bearing the Ehrlich ascites tumour. They find that glucuronic acid, or glucosamine and glucuronic acid, if administered in protracted doses, shorten the life of tumour-bearing mice (p. 362).

Csaba, Ács, Horváth and Kapa conclude, from *in vitro* and *in vivo* experiments, that tumours require heparinoid substances for their growth, and describe new possibilities for the inhibition of tumour growth (p. 367).

Lemon, Mueller, Looney, Chasen and Kelman have studied the citric acid concentration in blood from bone marrow, arterial and venous sites in healthy volunteers, arthritics and patients with various types of cancer. They find venous citric acid concentrations greater than twice the S.D. from the mean for each sex ("hypercitricaemia") in 4·2 per cent of healthy volunteers, in 4·6 per cent of all patients with benign diseases, in 11·1 per cent of patients with pre-malignant tumours, and in 23·6 per cent of cancer patients (p. 376).

ANOTHER GREAT ADVANCE

THE BACTIL

The new Bactil is the most up-to-date research microscope available. New in design, with a range of new accessories, including Phase Contrast equipment, the performance of the Bactil is unsurpassed.

Ten important new features, added to mechanical and optical excellence, characterise this outstanding instrument.

Write for List 101A.

W. WATSON & SONS LTD., BARNET, HERTS

AUTOMATIC COUNTING AND SIZING OF MICROSCOPIC MATERIAL

CASELLA ELECTRONICS

Casella Automatic Counter and Sizers take the tedium out of counting and sizing small particles, speed up the work, and improve accuracy. These instruments are being used to count and size inorganic powders and aerosols, using either the original material or shadows. Shadows are obtained by the evaporating aluminium technique, also they are being used in cellular biological research, notably in the field of cancer and bacteriology.

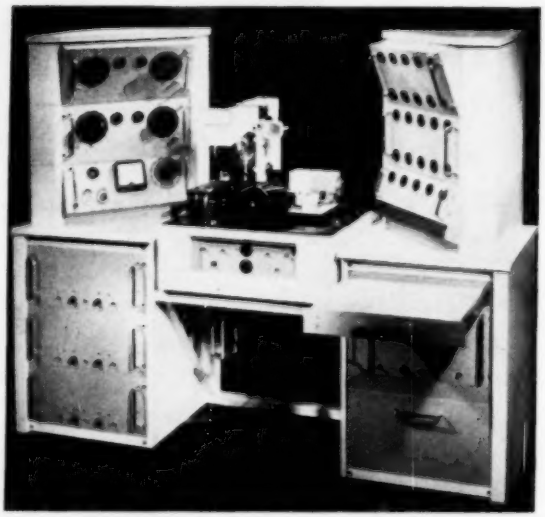
Scanning is by the wide track method. Using 200 scans of 10 mm. active regions per scan, an area of 50 mm.^{2} is covered in 55 seconds with the Console model and in 60 seconds with the Bench model. The size range covered is approximately 1.0 to 200μ. *The machines will be demonstrated in our showrooms to suit your convenience.*

BENCH MODEL

Embodies the same principles as the Console model but handles a smaller programme of sizing and counting. It is divided into two sections, comprising the mechanical and optical section and the electronics section.
Send for Brochure 906.

CONSOLE MODEL *(illustrated)*

This is a larger version of the Bench model and is designed for large programmes of sizing. It is fitted into a console cabinet for easy operation and can have up to five counting channels. Thus a size distribution into 12 sizes can be carried out in 30 minutes.
Send for Brochure 872.



CASELLA (ELECTRONICS) LTD., HAXBY ROAD, YORK. Telephone: 24112

Now controlled by Cooke, Troughton & Simms Ltd. Members of the Vickers Group.

The NEW MARCONI
OVERHEAD SUSPENSION MOUNTING
for Deep Therapy Techniques

- Complete freedom from obstruction at floor level
- Power operated self-locking vertical movement
- Manual or power operated horizontal movements
- Especially suitable for rotating therapy techniques

MARCONI INSTRUMENTS

SPECIALISTS IN THERAPEUTIC AND DIAGNOSTIC X-RAYS

Please address enquiries to MARCONI INSTRUMENTS LTD. at your nearest office:

London and the South: Marconi House, Strand, London, W.C.2 Telephone: COVent Garden 1234

Midlands: Marconi House, 24 The Parade, Leamington Spa. Telephone: 1408

North: 23/25 Station Square, Harrogate Telephone: 67455

Export Department: Marconi Instruments Ltd., St. Albans, Herts. Telephone: St. Albans 56161

XR40

Respiratory Disease
and the
General Practitioner

C. H. C. TOUSSAINT,
MRCS, LRCP, DPH

Consultant Chest Physician, Willesden Chest Clinic and Central Middlesex Hospital, London.

A survey of the diagnosis and management of respiratory disease, with detailed descriptions of 32 individual case histories, illustrated by 53 X-ray plates. Stress is laid upon early diagnosis of bronchial carcinoma, and a section on bronchitis is included.

28 pages *7s 6d*

From any bookseller or direct from :

THE CHEST AND HEART ASSOCIATION

Tavistock House North, Tavistock Square, London, W.C.1.

British Empire Cancer Campaign

A SURVEY OF CANCER IN LONDON

REPORT OF THE CLINICAL CANCER RESEARCH COMMITTEE

by W. L. HARNETT,
C.I.E., M.D., F.R.C.S,
Medical Secretary to the Committee.
With a **FOREWORD** by the
RT. HON. LORD HORDER,
G.C.V.O., M.D., F.R.C.P.,
and an **INTRODUCTION** by
SIR HENEAGE OGILVIE,
K.B.E., D.M., M.Ch., F.R.C.S.

Pp. vi + 834 with 22 figures in the text.

Price bound in paper covers 45/–, cloth 50/–.
Packing and postage,
Inland 3/9d, Canada 5/9, U.S.A. 7/6d.

BRITISH EMPIRE CANCER CAMPAIGN
11 Grosvenor Crescent, London, S.W.1

RATES FOR

ADVERTISEMENTS

IN THIS JOURNAL

Whole page	...	**£10 0 0**
Half page	...	**£5 10 0**
Quarter page	...	**£3 0 0**

Series discount 10%

All communications respecting Advertisements should be addressed to—

Mr. H. A. Collins
7 Lytton Gardens
Wallington, Surrey

Telephone: Wallington 1393

Niamid†

brand of nialamide

the safe monoamine oxidase inhibitor which offers

- relief in inoperable malignant states and other conditions associated with chronic intractable pain
- specific therapy for depressive states
- control of angina pectoris
- socialisation of certain types of mental defect

Niamid modifies and regulates levels of essential monoamines, widely distributed throughout body tissue. This action may explain its well-accepted clinical application in apparently unrelated conditions.

In inoperable malignant states the initiation of Niamid therapy can delay the introduction of narcotics. Even when their use is already established, a considerable reduction in dosage can be effected. Depressive states of all types have responded to the smooth gentle action of Niamid. Attacks of angina pectoris have been reduced in frequency and severity, and in certain types of mental defect there has been an improvement in social behaviour. Generally there is an uplift in mood and well-being, often accompanied by an increase in appetite.

Intensive studies and clinical trials have proved Niamid to be non-toxic. Niamid can be used with confidence in both ambulant and hospitalized patients.

Niamid is supplied in 25 and 100 mg. tablets.

Niamid

Niamid is marketed in the United Kingdom by

HARVEY PHARMACEUTICALS

a department of Pfizer Ltd., Folkestone.

HM26/3846

† Trade Mark

PRINTED BY ADLARD AND SON, LIMITED, BARTHOLOMEW PRESS, DORKING.

Respiratory Disease
and the
General Practitioner

C. H. C. TOUSSAINT,
MRCS, LRCP, DPH

Consultant Chest Physician, Willesden Chest Clinic and Central Middlesex Hospital, London.

A survey of the diagnosis and management of respiratory disease, with detailed descriptions of 32 individual case histories, illustrated by 53 X-ray plates. Stress is laid upon early diagnosis of bronchial carcinoma, and a section on bronchitis is included.

28 pages *7s 6d*

From any bookseller or direct from:

THE CHEST AND HEART ASSOCIATION

Tavistock House North, Tavistock Square, London, W.C.1.

British Empire Cancer Campaign

A SURVEY OF CANCER IN LONDON

REPORT OF THE CLINICAL CANCER RESEARCH COMMITTEE

by W. L. HARNETT,
C.I.E., M.D., F.R.C.S.,
Medical Secretary to the Committee.
With a FOREWORD by the
RT. HON. LORD HORDER,
G.C.V.O., M.D., F.R.C.P.,
and an INTRODUCTION by
SIR HENEAGE OGILVIE,
K.B.E., D.M., M.Ch., F.R.C.S.

Pp. vi + 834 with 22 figures in the text.

Price bound in paper covers 45/-, cloth 50/-,
Packing and postage,
Inland 3/9d. Canada 5/9, U.S.A. 7/6d

BRITISH EMPIRE CANCER CAMPAIGN
11 Grosvenor Crescent, London, S.W.1

RATES FOR

ADVERTISEMENTS

IN THIS JOURNAL

Whole page	...	£10 0 0
Half page	...	£5 10 0
Quarter page	...	£3 0 0

Series discount 10%

All communications respecting Advertisements should be addressed to—

Mr. H. A. Collins
7 Lytton Gardens
Wallington, Surrey

Telephone: Wallington 1393

Niamid†

brand of nialamide

the safe monoamine oxidase inhibitor which offers

- relief in inoperable malignant states and other conditions associated with chronic intractable pain
- specific therapy for depressive states
- control of angina pectoris
- socialisation of certain types of mental defect

Niamid modifies and regulates levels of essential monoamines, widely distributed throughout body tissue. This action may explain its well-accepted clinical application in apparently unrelated conditions.

In inoperable malignant states the initiation of Niamid therapy can delay the introduction of narcotics. Even when their use is already established, a considerable reduction in dosage can be effected. Depressive states of all types have responded to the smooth gentle action of Niamid. Attacks of angina pectoris have been reduced in frequency and severity, and in certain types of mental defect there has been an improvement in social behaviour. Generally there is an uplift in mood and well-being, often accompanied by an increase in appetite.

Intensive studies and clinical trials have proved Niamid to be non-toxic. Niamid can be used with confidence in both ambulant and hospitalized patients.

Niamid is supplied in 25 and 100 mg. tablets.

Niamid

Niamid is marketed in the United Kingdom by

HARVEY PHARMACEUTICALS

a department of Pfizer Ltd., Folkestone.

HM26/3846

† Trade Mark

PRINTED BY ADLARD AND SON, LIMITED, BARTHOLOMEW PRESS, DORKING.

THE BRITISH JOURNAL OF CANCER.

VOLUME XIV, No. 2 JUNE, 1960

CONTENTS

BODDINGTON, M. M., COWDELL, R. H. AND SPRIGGS, A. I.—Development of Carcinoma of the Cervix Uteri. Observations Resulting from Cytological Examination of 10,000 Cervical Smears 151

MARTINS, A. G.—Adenocarcinoma of the Uterus in Infancy 165

HOLLAND, R. H., ACEVEDO, A. R. AND CLARK, D. A.—The Arsenic Content of Bronchial Mucosa and Submucosa in Man. A Comparison of Specimens from Lung Cancer Victims and Control Tissue 169

BASERGA, R., PUTONG, P. B., TYLER, S. AND WARTMAN, W. B.—The Dose-response Relationship Between the Number of Embolic Tumor Cells and the Incidence of Blood-borne Metastases 173

HEWITT, H. B. AND WILSON, C. W.—Further Studies Relating to the Implications of Radiation Survival Curve Data for Treatment of CBA Mouse Leukaemia by Whole-body Irradiation 186

BIELSCHOWSKY, F. AND BIELSCHOWSKY, MARIANNE.—Carcinogenesis in the Pituitary Dwarf Mouse. The Response to 2-Aminofluorene 195

PEARSON, A. E. G.—Serial Irradiation of Mouse Tumours: Some Changes in Histology and Cytology 200

ISRAEL, M. S. AND ELLIS, I. ROSEMARY.—The Neoplastic Potentialities of Mouse Thyroid under Extreme Stimulation 206

GHADIALLY, F. N.—Carcinogenesis in the Skin of the Hedgehog. 212

LOUIS, C. J.—Flourescein-globulin Affinities of the Shope Papilloma 216

BELCHER, E. H. AND SIMPSON, SHIRLEY M.—Radioactive Tracer Studies of Red Cell Destruction in Rats bearing a Transplantable Tumour 224

MILLER, J. F. A. P.—Studies on Mouse Leukaemia. The Fate of Thymus Homografts in Immunologically Tolerant Mice 244

MODY, JER K.—The Action of Four Carcinogenic Hydrocarbons on the Ovaries of IF Mice and the Histogenesis of Induced Tumours 256

PULLINGER, B. D. AND IVERSEN, S.—Mammary Tumour Incidence in Relation to Age and Number of Litters in C_3H_f and $RIII_f$ Mice 267

PULLINGER, B. D.—Tests for Mammary Tumour Agent in C_3H_f and $RIII_f$ Mouse Strains 279

YOUNG, STRETTON AND FRASER, LUCY E.—Experiences with Hypophysectomy in Mice. Criteria of Complete Removal 285

DAVIES, W. AND WILMSHURST, J. R.—Carcinogens formed in the Heating of Foodstuffs. Formation of 3,4-Benzopyrene from Starch at 370–390° C. . . . 295

STEIN-WERBLOWSKY, RACHEL.—Induction of Cancer of the Cervix Uteri in Relation to the Oestrus Cycle 300

DRAYTON, H. A.—The Effect of Incubation on Three Fowl Tumour Viruses . 306

ALLISON, A. C. AND ARMSTRONG J. A.—Abnormal Distribution of Nucleic Acid in Tissue Culture Cells Infected with Polyoma Virus 313

MONTEMURRO, D. G.—The Effect of Nitrofurazone on the Testes and Accessory Sex Organs of Normal Rats and Rats bearing the Walker Carcinoma 256 319

CLARK, CATHERINE M. AND GOODLAD, G. A. J.—The Influence of Diet on the Action of the Walker 256 Carcinoma on Liver Protein and Nucleic Acids . 327

O'GORMAN, PATRICK.—The *In Vitro* Cytotoxic Activity of Murine Iso-antisera on Normal and Malignant Cells 335

MIRVISH, S. AND GILLMAN, J.—Bile Acid Composition and Bile Volume in Butter Yellow Fed Rats in Relation to Liver Cancer 346

CSABA, G., HORVÁTH, CECILIA AND ÁCS, TH.—Some New Data concerning the Biology of Tumours. The Effects of Heparin and its Components on Tumour Growth 362

CSABA, G., ÁCS, TH., HORVÁTH, CECILIA AND KAPA, ESZTER.—Some New Data concerning the Biology of Tumours. The Effects of Heparin Inhibitors on Tumour Growth 367

LEMON, H. M., MUELLER, J. H., LOONEY, J. M., CHASEN, W. H. AND KELMAN, MARCIA.—Hypercitricemia in Human Cancer. Factors concerned in Pathogenesis and Treatment 376

SURVEY OF PAPERS . . . . . (i)